University of Alberta

Characterization of a Novel Nuclear Import Pathway Mediated by the Yeast Karyopherin Kap104p

by

Dennis C.Y. Lee ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science

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Abstract

Kap104p is a *Saccharomyces cervisiae* nuclear import receptor (karyopherin) for two essential mRNA-binding proteins, Nab2p and Nab4p/Hrp1p. This thesis demonstrates that Kap104p binds directly to its substrates separately and independently through similar arginine/glycine rich (RG-rich) domains. It is shown here that these RG-rich domains also serve as *KAP104* dependent nuclear localization signals (NLS) *in vivo*, and will be termed RG-NLS. Like other karyopherins, Kap104p interacts with the small GTP-binding protein Ran. Ran in its GTP bound form (Ran-GTP) in combination with RNA stimulates release of Nab2p or Nab4p from Kap104p. On the other hand, the addition of Kap104p to a pre-formed complex of Nab2p or Nab4p with single stranded DNA–cellulose results in the displacement of the Nab from the column. Kap104p is also able to directly bind to repeat containing nucleoporins and this association is destabilized in the presence of Ran-GTP alone. In addition, Kap104p can form a trimeric complex with RanBP1 and Ran-GTP or –GDP, which can be dissociated by the addition of Nab4p/Hrp1p. The data collected here can be compiled to form a model for Kap104p-mediated nuclear import.

This Thesis is dedicated to my parents, Dr. Sho Tone Lee and Mrs. Ivy Chen Huan Lee.

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Abbreviations

cNLS, classical nuclear localization signal

DNA, deoxyribonucleic acid

GDP, guanosine diphosphate

GFP, green fluorescent protein

GST, glutathione S-transferase

GT, glutathione

GTPγS, gunaosine 5'-3-*O*-(thio)triphosphate

GTP, guanosine triphophate

HIV-1, human immunodeficiency virus type1

hnRNP, heterogeneous nuclear ribonuclearprotein

kap,ᵝkaryopherin

MBP, maltose binding protein

mRNA, messenger ribonucleic acid

Nab, nuclear poly(A)-binding protein

NES, nuclear export signal

NPC, nuclear pore complex

NRS, nuclear retention sequence

NTF2, nuclear transport factor 2

Nup, nucleoporin

PAGE, polyacrylamide gel electrophoresis

Ran, ras-related nuclear protein

RanBP1, Ran binding protein 1

RanGAP, ran GTPase activating protein

RCC1, regulator of chromosome condensation 1

RG-NLS, arginine/glycine-rich NLS

RNA, ribonucleic acid

RRM, RNA recognition motif

rRNA, ribosomal ribonucleic acid

SRP1, suppressor of RNA polymerase I

ssDNA, single stranded DNA

SV40lT, Large tumor antigen from Simian Virus 40

tRNA, transfer ribonucleic acid

Yrb1, yeast ran binding protein 1

1. Introduction

1.1. General Nuclear Pore Complex structure

In eukaryotic cells, compartmentalization of DNA replication/transcription and protein synthesis creates a need for the transport of proteins, and nucleic acids between the nucleus and cytoplasm. The nucleus is enclosed by the nuclear envelope, a double membrane structure that is continuous with the endoplasmic reticulum, and forms a physical barrier that separates the nucleoplasm from the cytoplasm. Nucleocytoplasmic transport of biological molecules occurs through gated channels embedded in the nuclear envelope known as nuclear pore complexes (NPCs) (reviewed by Rout and Wente, 1994; Nigg, 1997). NPCs are massive cylindrical complexes composed of nuclear pore proteins called nucleoporins or nups. Yeast NPCs have a molecular mass of ~66 megadaltons (Rout and Blobel, 1993), while higher vertebrates possess larger NPCs with molecular mass of ~125 megadaltons (Reichelt et al., 1990). NPCs contain eight symmetrical spokes radiating around a central core. These spokes are attached to two rings which run parallel to the plane of the nuclear envelope. Eight filaments extend from the cytoplasmic face of the pore into the cytoplasm. Similarly, eight filaments extend from the nuclear face of the pore into the nucleoplasm, and terminate at a structure termed the basket ring (see Rout and Wente, 1994; Yang et al., 1998). The aqueous central channel of the NPC allows passive diffusion of ions, metabolites, and small proteins (~40 kD) (Rout and Wente, 1994; Gorlich and Mattaj, 1996). However, most proteins and RNA/protein complexes are too large to passively diffuse through the NPC. Instead, these molecules are actively transported through the pore via specific saturable and energy dependent mechanisms which require mobile, soluble transport proteins (Gorlich and Mattaj, 1996; Nigg, 1997).

1.2. Nuclear Localization Signals and heterodimer import

Proteins destined for the nucleus bear a nuclear localization signal (NLS) (Dingwall and Laskey, 1991), whereas molecules exiting the nucleus contain nuclear export signals (NES) (Gerace, 1995; Fischer et al., 1996). These signals are recognition sites for transport factors which ultimately mediate the translocation of substrates through the nuclear pore. Although there is not a strict consensus sequence which encompasses

all known nuclear targeted proteins, there are two common features which many substrates possess. First, the actual length of the sequence is generally less than twelve amino acids and second, the sequence contains a high proportion of positively charged (basic) amino acids (Dingwall and Laskey, 1991). The most well characterized NLS is from the simian virus (SV) 40 Large T antigen (SV40lT). Studies have shown that a highly basic sequence within SV40lT is necessary for its nuclear import, and mutations in this sequence prevent the protein from being localized to the nucleus (Kalderon et al., 1984a; Lanford and Butel, 1984). Also using chimeras, the minimal sequence within SV40lT required to promote nuclear localization of a normally non-nuclear protein, pyruvate kinase, was determined to be PKKKRKV (Kalderon et al., 1984b). SV40lT is regarded as a model for identifying potential NLSs via sequence homology, and has led to the identification of many signals carried on nuclear proteins. Collectively these short, basic, signals are referred to as classical nuclear localization signals (cNLSs) (Wozniak et al., 1998) and require the same import machinery, which will be discussed later.

As mentioned above, movement of molecules in and out of the nucleus requires specific proteins that function as transporters. Whether participating in nuclear import or export these proteins are collectively termed karyopherins [karyon from the Greek word for nucleus, and pher(ein) meaning "to bring to" or "to carry from" (Radu et al., 1995a)] or kaps (reviewed by Wozniak et al., 1998). The first karyopherins to be identified were responsible for importing cNLS bearing substrates. Two proteins, kap α (Radu et al., 1995a) (importin α (Gorlich et al., 1994), NLS receptor (Adam and Gerace, 1991; Adam and Adam, 1994)), and kap β (Radu et al., 1995a) (importin β (Gorlich et al., 1995a), p97 (Chi et al., 1995)), form a heterodimeric complex which mediates nuclear import. The α subunit is responsible for NLS binding, while the β subunit docks the NLS/α/β complex to the NPC (Adam and Gerace, 1991; Gorlich et al., 1995b; Morianu et al., 1995; Radu et al., 1995a; Weis et al., 1995). It is proposed that a series of docking and release steps facilitate movement of the import complex through the NPC into the nucleus (Radu et al., 1995b; Rexach and Blobel, 1995).

In *Xenopus*, two α subunits α_1(54 kD) and α_2 (56 kD), and one associated β (97 kD) subunit have been identified (Radu et al., 1995a). Similarly, two αs and one β with the same molecular masses as in *Xenopus* have been characterized in bovine erythrocytes (Adam and Gerace, 1991; Adam and Adam, 1994; Chi et al., 1995). In contrast to these findings only one α and one β subunit have been found in the yeast *Saccharomyces cervisiae*. Initially yeast kap α was identified as a suppressor of certain temperature sensitive mutants of RNA polymerase I, and named SRP1 for **s**uppressor of **R**NA **p**olymerase **I** (Yano et al., 1992). Later studies revealed that Srp1p is essential for nucleolar structure, RNA transcription, and microtubule function (Yano et al., 1994), and was localized to the nuclear envelope at or around the NPC (Yano et al., 1992). Immuno-isolation of protein A tagged Srp1p (Kap60p, 60 kD) revealed a complex between Kap60p and a 95 kD protein (Kap95p) which showed 33% identity and 63% similarity to rat kap β (Enenkel et al., 1995). This heterodimer was also able to direct docking of a cNLS bearing protein to the nuclear pore in mammalian cells (Enenkel et al., 1995). *In vitro* studies have revealed that Kap60p functions like kap α in vertebrates by binding to the cNLS of nuclear bound proteins, and Kap95p, like kap β in vertebrates, is responsible for docking the import complex to the NPC (Rexach and Blobel, 1995). Rexach and Blobel (1995) have also shown that cNLS binding by Kap60p was enhanced in the presence of Kap95p, demonstrating the cooperative nature of the formation of an import complex.

1.3. Other transport pathways

Not all proteins destined for the nucleus carry a cNLS, thus they do not utilize the α/β heterodimer as the main mechanism for nuclear localization. In *Saccharomyces cervisiae*, sequence comparison of the yeast genome with Kap95 suggests that the yeast kap family may contain as many as 14 members (Mattaj and Englmeier, 1998; Pemberton et al., 1998; Wozniak et al., 1998). The first kap β related proteins shown to function in nuclear transport were the yeast and mammalian proteins Kap104p (Aitchison et al., 1996) and karyopherin β_2 (kap β_2) or Transportin (Trn) (Pollard et al., 1996; Bonifaci et al., 1997). Both transporters import mRNA binding proteins that do not contain a cNLS.

Kap104p is responsible for importing two **h**eterogeneous **n**uclear **r**ibo**n**uclear

proteins (hnRNPs) Nab2p and Nab4p/Hrp1p1 (Aitchison et al., 1996). Nab4p/Hrp1p (Henry et al., 1996), is the most similar yeast hnRNP in sequence to mammalian hnRNP A1 and plays a role in mRNA 3' end formation (Kessler et al., 1997). Like hnRNP A1, Nab4p/Hrp1p is able to shuttle between the nucleus and cytoplasm (Kessler et al., 1997). The exact function of Nab2p has yet to be determined, however Nab2p has been shown to interact with polyadenylated RNA and is likely part of an hnRNP complex which exits the nucleus (Anderson et al., 1993). Similar to Kap104p, kap β_2 also imports hnRNP proteins, and was initially identified by its ability to directly interact with a 38 amino acid residue sequence termed M9 found within hnRNP A1; and mediate its import (Pollard et al., 1996). The M9 sequence bears no resemblance to a cNLS, is necessary for the nuclear localization of hnRNPA1, and is sufficient to target non-nuclear proteins to the nucleus (Siomi and Dreyfuss, 1995). Interestingly, M9 can also function as an NES and is able to shuttle between the nucleus and cytoplasm (Michael et al., 1996; Bogerd et al., 1999).

1.4. Role of nucleoporins

Upon recognition and binding of transport substrates by their respective karyopherins, import or export complexes are thought to dock at the NPC. As mentioned above, the NPC is composed of nucleoporins (nups), which facilitate translocation through the pore (Rout and Wente, 1994). Although the exact role of nucleoporins in transport is not entirely known, certain pore proteins are directly involved. Of particular interest are nucleoporins that contain a degenerate repeated dipeptide, phenylalanine-glycine (FG). These so called FG nups can be further subdivided into proteins in which the repeats form a tetrapeptide containing FXFG or GLFG (using amino acid single-letter code, and X referring to any amino acid) (Rout and Wente, 1994; Nigg, 1997). These repeat containing nucleoporins are able to bind karyopherins *in vitro* through these repeat sequences (Iovine et al., 1995; Radu et al., 1995b; Rexach and Blobel, 1995; Aitchison et al., 1996; Stutz et al., 1996). Kap104p interacts with the GLFG repeat containing nucleoporins Nup116p, Nup100p, Nup145p, and Nup57p, and displays weak interactions with the FXFG repeat of Nup1p (Aitchison et al., 1996). Kap95p has affinity for Nup1p,

Nsp1p, as well as Nup116p, Nup100p, Nup145p, and Nup57p, however the α/β heterodimer does not bind to the GLFG repeat of Nup145, whereas binding to the FXFG of Nup1p is strong (Rexach and Blobel., 1995; Iovine et al., 1997). It is still not certain whether all karyopherins have varying affinities for specific nucleoporins, or if these apparently different binding affinities represent different docking sites utilized *in vivo*. However, based on the ability of karyopherins to interact with multiple nucleoporins, it has been proposed that that translocation through the pore occurs along an affinity gradient composed of multiple karyopherin docking sites (Rexach and Blobel., 1995).

Besides repeat containing nucleoporins, studies have shown that non-repeat containing nucleoporins may also be invovled in nucleocytoplasmic transport. Nup53p is a repeat nucleoporin that can be isolated in a nuclear pore sub complex with Nup59p, Nup170p, and the transport factor Kap121p. Nup53p binds strongly to Kap121p *in vitro* and shows specificity to only this karyopherin (Marelli et al., 1998). Nup170p is not a repeat containing nucleoporin, however may play a role in transport via interactions with Nup53p and Nup59p (Marelli et al., 1998). Other non-repeat containing nucleoporins such as Nup120p, Nup84p, and Nup85p form another sub complex that has been suggested to play a role in mRNA export (Aitchison et al., 1995b; Siniossoglou et al., 1996). Thus participation in nuclear transport is not restricted to repeat containing nucleoporins.

1.5. Role of Ran in transport

In addition to transporters, the small GTPase Ran (**ra**s-related **n**uclear protein) and its interacting protein, p10/NTF2 (**n**uclear **t**ransport **f**actor 2), play a critical role in nuclear import and export (Moore and Blobel, 1993; Moore and Blobel, 1994; Melchior et al., 1995; Paschal and Gerace, 1995; Nehrbass and Blobel, 1996; Cole and Hammell, 1998; Moore, 1998). Ran is a G protein that cycles between GDP and GTP bound states, and is primarily located in the nucleus (Moore, 1998) where it is believed to be maintained in a GTP-bound state by the nuclear-restricted GTP exchange factor RCC1 (**r**egulator of **c**hromosome **c**ondensation 1) (Seki et al., 1996). In contrast the localization

of RanGAP (Ran GTPase activating protein) to the cytoplasm and the cytoplasmic filaments of mammalian NPCs (Matunis et al., 1996; Mahajan et al., 1997) is thought to ensure cytoplasmic Ran is maintained in a GDP-bound form. This separation of Ran creates two different environments which provide specificity and directionality for the binding and release steps that occur during translocation through the NPC (Gorlich and Mattaj, 1996; Cole and Hammell, 1998; Moore, 1998). For example, the formation of an import complex containing kaps α/β and a cNLS is stable in the presence of Ran-GDP, but Ran-GTP triggers its disassembly (Rexach and Blobel, 1995; Percipalle et al., 1997). In contrast, export complexes consisting of an NES-containing protein and its export factor, Crm1p/Exportin 1, are stabilized by Ran-GTP in a trimeric complex (Fornerod et al., 1997). These mechanisms are thought to ensure that cargoes are loaded and released from their appropriate carriers in the correct locations.

The exact energy mechanism needed to drive translocation is not clearly understood, however GTP hydrolysis on Ran is not required for (at least) a single round of either import or export *in vitro* (Richards et al., 1997; Schwoebel et al., 1998; Englmeier et al., 1999; Ribbeck et al., 1999). Instead nuclear Ran-GTP is needed for unloading substrates and recycling transport factors back to the cytoplasm (Ribbeck et al., 1999). The Ran associated factor p10 in yeast (NTF2 in humans) has been shown to be required for translocation of import complexes through the pore (Moore and Blobel, 1994; Paschal and Gerace, 1995; Nehrbass and Blobel, 1996). In addition NTF2 has been shown to mediate the import of Ran into the nucleus (Ribbeck et al., 1998; Smith et al., 1998).

Studies have shown that p10 is responsible for docking Ran-GDP to the pore and forms a pentameric complex with Kap95p/Kap60p/Ran-GDP and the FG repeats of Nup36p (ΔNup36p) through interactions with Kap95p, Ran-GDP, and ΔNup36p (Nehrbass and Blobel, 1996). The exact function of p10 has yet to be determined, however p10 has been suggested to coordinate Ran and ensure spatial and temporal separation of association and disassociation events during import (Nehrbass and Blobel, 1996).

1.6. Function of RanBP1p

Ran-GAP stimulated GTPase activity of Ran is inhibited by binding to kap β or transportin/kap β_2 (Floer and Blobel, 1996; Bischoff and Gorlich, 1997; Lounsbury and Macara, 1997). Thus it would seem that as karyopherins exit the nucleus bound to Ran-GTP, a mechanism promotes the hydrolysis of GTP so that the kap may return to its original state. A protein termed Ran **B**inding **P**rotein 1 (RanBP1p) in mammals (Coutavas et al., 1993), **Y**east **R**an **B**inding Protein 1 (Yrb1p) in *Saccharomyces cervisiae* (Schlenstedt et al., 1995) appears to fulfill this function by stimulating Ran-GTP hydrolysis by increasing the affinity of Ran-GTP for Ran-GAP (Bischoff et al., 1995; Bischoff and Gorlich, 1997; Lounsbury and Macara, 1997). RanBP1p can form a trimeric complex with kap β/Ran-GTP (Lounsbury and Macara, 1997), and also kap β_2/RanGTP (Bischoff and Gorlich, 1997). This may be an intermediate complex that forms as the kaps exit the nucleus bound to Ran-GTP and encounter RanBP1p on the cytoplasmic face of the pore. However, there are conflicting views on whether the complex remains intact after GTP hydrolysis. In one instance a stable complex can be formed *in vitro* between kapβ/Ran-GDP/RanBP1p (Chi et al., 1997). In contrast, other studies have demonstrated that the addition of RanBP1p to a kapβ/Ran-GTP, or kapβ_2/Ran-GTP results in a trimeric complex which dissociates upon GTP hydrolysis. In the former case kap α, in combination with RanBP1p, stimulates hydrolysis (Bischoff and Gorlich, 1997). Thus it is not clear whether RanBP1p forms a trimeric complex with kaps bound to RanGDP in the cytoplasm, however it is a critical factor in recycling transport factors for additional rounds of transport.

1.7. RNA export and hnRNPs

The majority of nuclear encoded RNAs, such as mRNA, tRNA, and rRNA are exported to the cytoplasm. The exact mechanism of transport is still not clear, however recent work suggests that RNA is exported as part of an hnRNP complex (reviewed by Nakielny and Dreyfuss, 1997). It is thought that some of these RNPs serve as adapters by directly binding RNA and mediating export. The **h**uman **i**mmunodeficiency **v**irus type1

(HIV-1) regulatory protein Rev is essential for nuclear export of partially spliced and unspliced viral transcripts (Chang and Sharp, 1989; Emerman et al., 1989; Febler et al., 1989; Malim et al., 1989). Rev contains two functional domains that are necessary for its function in RNA export. Firstly, the N-terminus of Rev contains an arginine-rich RNA binding domain that recognizes an RNA element found on all HIV transcripts, termed **R**ev **r**esponse **e**lement (RRE). This domain is also responsible for Rev import and multimerization. Secondly, the C-terminus harbors a leucine-rich NES which has been shown to be a substrate for the yeast nuclear export receptor CRM1 (Dreyfuss et al., 1993; Fischer et al., 1996; Fornerod et al., 1997; Nakielny and Dreyfuss, 1997). The two domains together mediate the export of viral mRNA from the nucleus.

Recent studies have shown that yeast and higher eukaryotes contain RNPs which which may serve analogous functions to Rev. Numerous RNA binding proteins have been identified which posses the ability to shuttle between the nucleus and cytoplasm and are candidates for mediating RNA export (Pinol-Roma and Dreyfuss, 1992; Dreyfuss et al., 1993; Fischer et al., 1996; Nakielny and Dreyfuss, 1997). HnRNP A1 is one of the most abundant RNP proteins in mammalian cells, and has been shown to be essential for mRNA export (Dreyfuss et al., 1993; Izaurralde et al., 1997a). It is likely that hnRNP A1, like Rev, acts as an adapter for mRNA export by directly binding to mRNA and accompanying the hnRNP/mRNA complex to the cytoplasm (Nakielny and Dreyfuss, 1997; Izaurralde et al., 1997a) where it can be recognized by kap β_2 and re-imported into the nucleus (Pollard et al., 1996; Bonifaci et al., 1997). The major characteristics of hnRNP A1 are two RNA binding domains located in the N-terminus, a stretch rich in arginine and glycine amino acid residues (RGG box) (Kiledjian, 1992) in the central region, and a glycine-rich C-terminus termed M9 (Dreyfuss et al., 1993; Siomi and Dreyfuss, 1995). Other RNP proteins such as hnRNP A2, B1 also share the same functional domains as A1 and also shuttle between the nucleus and cytoplasm (Nakielny and Dreyfuss, 1997). In contrast hnRNP C1 and C2 are confined to the nucleus and do not shuttle (Nakielny and Dreyfuss, 1996). Interestingly, the C proteins contain a **n**uclear **r**etention **s**equence (NRS) which prevents them from exiting the nucleus even in the presence of an NES (Dreyfuss et al., 1993; Nakielny and Dreyfuss, 1996; Nakielny and

Dreyfuss, 1997). Thus RNAs and/or RNPs bound to NRS containing proteins are not exported. This may be an important mechanism to prevent the export of immature RNA, which would be disadvantageous to the cell.

1.8. Objectives of the Thesis

In yeast, Nab2p and Nab4/Hrp1p are also nuclear mRNA binding proteins and are essential for post-transcriptional processing and export of mRNA (Anderson et al., 1993; Wilson et al., 1994; Henry et al., 1996; Kessler et al., 1997). As mentioned above, these RNPs are substrates for Kap104p, which mediates their import into the nucleus (Aitchison et al., 1996). To further understand the function of Kap104p in nuclear transport, this thesis will characterize the interactions between Kap104p and Nab2p and Nab4p/Hrp1p using *in vitro* and *in vivo* approaches. Previous studies have established that Kap104p is able to interact with repeat containing nucleoporins (Aitchison et al., 1996). These initial observations have invoked the question of what role do these nucleoporins play in Kap104p mediated import. To address this, molecular approaches were implemented using recombinant proteins. As seen in the case for Kap60p/Kap95p and kap α/β mediated transport, the small GTPase Ran plays a critical role in cNLS import (Moore and Blobel, 1993; Melchiro et al., 1995; Rexach and Blobel, 1995; Percipalle et al., 1997; Moore, 1998). The importance of Ran in Kap104p mediated transport was also investigated, as well as the possible function of RanBP1p. Furthermore it is not clear whether Kap104p is also involved in RNA export. A novel *in vitro* assay was developed to directly assess the possible role of Kap104p in RNP mediated RNA export. The evidence collected can be assembled into a simple model that characterizes Kap104p mediated nuclear import.

2. Materials and Methods

2.1. Gene amplification and isolation

Genes of interest were amplified via the polymerase chain reaction (PCR) from the yeast genome using 1 µl of Taq DNA Polymerase (1U/µl of Expand HF Boehringer Mannheim, Quebec), 0.8 µl dNTP mixture (0.2 mM of each nucleotide)(NEB, Mississauga,Canada) 10 µl Taq Buffer (10X)(Boehringer Mannhein), 1 µl of each primer (1 µM final concentration)(Life Technologies, Burlington, Canada), and 0.5 µl *S.cervisiae* genomic DNA (214 µg/ml)(Promega, Madison, WI) in a 100 µl final volume. Reactions were run following standard procedures. Amplified products were treated with 1 volume of chloroform (BDH Inc., Toronto, Canada), and centrifuged for 3 minutes at 11 250 x g at 4°C. Samples were then precipitated with 2.5 volumes of 100% ethanol (Commercial Alcohols Inc., Brampton, Canada), 1/10th volume 3 M Sodium Acetate (pH 5.5), and 1 µl glycogen (Boehringer Mannhein) for 30 minutes at -20°C. Samples were then centrifuged for 30 minutes at 11 250xg, 4°C, washed once with 70% ethanol, dried and resuspended in water. PCR products were digested with the required restriction enzymes in a final volume of 100 µl, and incubated at 37°C over night. Simultaneously, the necessary plasmid was also digested in the same fashion. Insert and plasmid samples were then centrifuged under vacuum at 65°C, for 20 minutes to reduce sample volume before being run on a 1% GTG agarose gel (FMC, Rockland, ME) in 1% TBE (Tris-Borate EDTA). Desired bands were then excised under long wave UV and placed into a unidirectional electroelution chamber (International Biotechnologies Inc., New Haven, CT) containing 0.5% TBE which was pre-loaded with 80 µl of 7.5 M Ammonium Acetate + Bromo-phenol-blue in each running well. DNA elution was performed at 100 Volts for 1 hour at room temperature. The chamber was then drained and wells containing the salt/DNA mixture were removed (~400 µl), and precipitated with 1 ml of 100% ethanol and 1 µl of glycogen as above. Pellets were then dried under vacuum at room temperature for 5 minutes, and resuspended in 10 µl of water.

2.2. Ligation of insert DNA into cloning vectors

Ligation reactions were done in a 10 µl final volume with 1 µl T4 DNA ligase (Boehringer Mannheim), and 1 µl T4 DNA ligase buffer (10X)(Boehringer Mannheim) at 16°C, over night at approximately 5:1 insert:vector molar ratio and 200 ng total DNA per 10µl reaction. In addition vector alone controls were also implemented to estimate ligation efficiency.

2.3. Bacterial Transformation

DNA of interest (1 µl for plasmid constructs, 5 µl for ligation reactions) was mixed with 100 µl of competent cells (see below) and placed on ice for 30 minutes. Cells were then heat shocked for 3 minutes at 37°C, and then immediately put on ice for 2 minutes. 900 µl of LB (Luria-Bertani) medium was added and the samples incubated at 37°C, for 1 hour with shaking. Cells were then pelleted and 900 µl of media removed. The remaining cells were resuspended in the residual media and plated on the desired media.

2.4. Plasmid extraction (mini-prep)

Three ml overnight cultures were inoculated with single colonies. 1.5 ml of culture was pelleted in a 1.5 ml tube. Media was aspirated and cell pellets resuspended in 100 µl TE + RNAase A (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNAase A), and placed on ice for 5 minutes. 200 µl of 0.2 M NaOH/1% SDS was added and tubes were inverted gently 20 times, and put on ice for 5 minutes. 150 µl of 3M Potassium (BDH) 5M Acetate (Fischer Scientific, Nepean, Canada) was added and tubes were inverted gently until a white precipitate was visible, and the solution became less viscous. Samples were then placed on ice for 10 minutes, and centrifuged at 4°C, 11 250 x g, for 10 minutes. The supernatant (~450 µl) was transferred to a fresh 1.5 ml tube and 450 µl of phenol:chloroform (Life Technologies) (BDH Inc.) mixture (50:50) added. Samples were vortexed for 5 seconds and centrifuged at 4°C, 11 250 x g, for 3 minutes. The aqueous top layer was transferred to a new tube and 450 µl of chloroform (BDH Inc.,

Toronto, Canada) was added. Samples were then mixed and centrifuged as above. The top aqueous phase was then transferred to a new tube and 1 ml of 100 % ethanol added. Tubes were inverted to mix, and incubated at -20°C for 30 minutes. Samples were then centrifuged at 4°C, 11 250 x g, for 30 minutes, washed with 70 % ethanol and re-centrifuged at 4°C, 11 250 x g, for 5 minutes. Pellets were dried using an Eppendorf 5301 concentrator and resuspended in 30 µl of TE + RNAase A (see above).

2.5. Yeast Transformation

5 ml overnight cultures were inoculated with the desired yeast strain. Cultures were then diluted by adding the 5 ml overnight culture to 45 ml of fresh media and grown for 5 hours at the desired temperature with shaking. Cells were pelleted at 4°C, 2268 x g, for 5 minutes, and the media poured off. Water (1 ml) was used to resuspend the cells, followed by centrifugation at 4°C, 1960 x g, for 1 minute. The supernatant was aspirated and the water wash was repeated. Cells were then washed twice as above with ice-cold 1M Sorbitol, and resuspended in a minimal volume (~40 µl) of ice-cold 1M Sorbitol. Samples were aliquoted into 45 µl portions and 1 µl of mini-prep DNA added. Cells were then loaded into pre-chilled electroporation cuvettes (0.2 cm gap width; BIORAD, Hercules, CA), and electroporated using a BIORAD Gene Pulser) set at 1.5 kV, 200 Ω, 25 µFD and immediately put into 150 µl pre-chilled 1M Sorbitol. The entire sample was then plated on the required selective media.

2.6. Plasmids and strains

Yeast strains were derived from DF5 (Mat a/Mat a ura3-52/ura3-52 his3Δ200/ his3Δ200 trp1-1/trp1-1 leu2-3,112/ leu2-3,112 lys2-801/lys2-801) or W303 (Mat a/Mat a ade2-1/ade2-1 ura3-1/ura3-1 his3-11,15/ his3-11,15 trp1-1/trp1-1 leu2-3,112/ leu2-3,112 can1-100/can1-100) (74). KAP104 strains are derivatives of DF5 cells and have been described (Aitchison et al., 1996). KAP95 temperature sensitive mutants were constructed by plasmid shuffling as described in Aitchison et al., 1996. KAP104-GAL cells are derivatives of W303 cells and were constructed by incorporating the GAL10 promoter downstream to a URA3 marker and upstream to the KAP104 initiation codon as described in Aitchison et al., 1995.

KAP104 and *NAB2* open reading frames (ORFs) were amplified from yeast genomic DNA by PCR, cloned into the BamHI site of pGEX-2TK (Pharmacia, Uppsala, Sweden) and termed Kap104-pGEX2TK and Nab2-pGEX2TK respectively. Likewise, *NAB4/HRP1* was cloned into the BamHI site of pGEX-4T1 and termed Nab4-pGEX4T1. The C-terminal deletion of Nab4p was constructed by digesting full length Nab4p-pGEX4T1 with NdeI and SmaI. T4 DNA polymerase was then used to create blunt ends and the linearized fragment was re-ligated, described above, but at a DNA concentration of 2ng/µl. *RANBP1* was also amplified from the yeast genome and cloned into the 5'BamHI and 3'EcoRI sites in pGEX2TK, and termed RanBP1-pGEX2TK. Kap95p-GST (Rexach and Blobel, 1995) were a gift from M. Rexach (Stanford University). Full length Nab2p tagged with Green Fluorescent Protein (GFP) was a gift from C. Guthrie (University of California at San Francisco). Nup116-pMAL, Nup100-pMAL, and GLFGp-GST (pGEX3X) of Nup116p were gifts from S.Wente (Washington University School of Medicine).

2.7. Nab2p and Nab4p deletion constructs

Nab2p deletion constructs Δ8-187, Δ200-254 and Δ303-499 were constructed by PCR using Nab2-pGEX2TK as a template. Oligonucleotides containing SacI restriction sites were designed to amplify *NAB2* and pGEX2TK, while omitting the desired site of insertion. The fragments were purified, digested with SacI and ligated as described above. Recombinant plasmids were sequenced to confirm deletions. Nab4p fragments encoding amino acid residues 492-534, 392-534 and 241-400 were amplified from yeast genomic DNA (Promega, Madison, WI) using PCR and oligonucleotides containing restriction sites for cloning as described below. Fragments were cloned into the 5'BamHI, 3'EcoRI sites in pGEX-2TK, or into the 5'HindIII, 3'EcoRI sites of NES-GFP2-NLS (pKW430) or p12-GFP2-NLS (pKW431)(Stade et al., 1997). PKW431 was a gift from K.Weis (University of California at San Francisco). In each case the cNLS was removed and replaced with *NAB2* or *NAB4/HRP1* coding sequence upstream of, and in frame with, a mutant (p12; nes) or wild-type NES followed by two GFP coding sequences (2XGFP). The C-terminal deletion of Nab4p was constructed by digesting full

length Nab4-pGEX4T1 with NdeI and SmaI. T4 DNA polymerase was then used to create blunt ends and the sample was ligated as described above.

2.8. GST expression check

Single colonies were inoculated into 3 ml of LB + 100 ug/ml Ampicillin (AMP)(Fischer Scientific) and grown overnight. Samples were then diluted by adding 1ml of the overnight to 2 ml of fresh LB + AMP, and grown for 30 minutes at 37°C with shaking. One ml of culture was pelleted, and put on ice. This was designated as the uninduced sample. The remaining cultures were then induced by the addition IPTG (isopropylthio-β-D-galactoside)(Life Technologies) to a final concentration of 2 mM, and grown at 37°C with shaking for 2 hours. One ml of induced culture was placed in a fresh tube and processed as described for the uninduced. Samples were then resuspended in 100 µl 50 mM Tris/20 mM EDTA, pH 8.0, followed by the addition of 50 µl 3X SDS sample buffer (NEB). Lysates were heated for 15 minutes at 65°C, and sonicated for 3 seconds using a Branson Sonifier 450, before analysis by SDS PAGE and Coomassie Blue staining. Clones which expressed the fusion protein of choice were used to prepare large scale protein lysates as below.

2.9. Recombinant Protein Preparation

GST fusion proteins were expressed separately in *E. coli* (BL21 DE3 pLYS-S) (Novagen, Madison, WI). Saturated cultures (5ml) were diluted by adding the overnight to 195 ml LB + AMP and grown for 3.5 hours at 37°C with shaking. Expression of the chimeras was induced by the addition of IPTG to a final concentration of 2 mM and the cells were incubated at 37°C for an additional 2 hours. Cells were harvested, washed with 30 ml of water and pellets were rapidly frozen with dry ice ethanol. Pellets were then resuspended in 30 ml of Transport Buffer (20 mM HEPES-KOH pH 7.4, 110 mM KOAc, 2 mM $MgCl_2$, 1 µM $CaCl_2$, 1 µM $ZnCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20) containing 300 µl of solution P (0.4 mg/ml Pepstatin A, 18 mg/ml phenylmethylsulfonyl fluoride(Aitchison et al., 1996) and immediately lysed by sonication using a Bronson Sonifier 450 (3X, 30 second constant output of 60). The resulting lysate was clarified by centrifugation at 2500 x g for 15 min, 4°C then 17,000 x

g for 20 min at 4°C. The lysates were frozen with dry ice ethanol, and stored at -80°C. For Nup116-pMAL and Nup100-pMAL, lysates were prepared the same as GST fusions except Column Buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) was used instead of Transport Buffer.

2.10. Ran preparation

Yeast Ran (Gsp1) (Floer and Blobel, 1996) was a gift from M. Floer and G. Blobel (The Rockefeller University, NY). Ran was loaded with 3 forms of guanine nucleotide (Sigma): GTP, GTPγS (guanylyl imidodiphosphate, non-hydrolyzable form of GTP) or GDP as described (Rexach and Blobel, 1995). A reaction mixture containing 30 mM EDTA, 4 mM DTT, 1.2 mM guanine nucleotide, in Binding Buffer (20 mM HEPES–KOH pH 6.8, 2 mM $MgOAc_2$, 150 mM KOAc,) was incubated with an equal volume of Ran (0.8 µg / µl) at room temperature for 90 minutes. Magnesium acetate was added to a final concentration of 30 mM, samples were incubated at 4°C for 15 minutes and stored at –80 °C.

2.11. *In Vitro* solution binding assay

Protein lysates (described above) were thawed in ice water and incubated with 20 µl of glutathione resin (GT; Pharmacia), pre-equilibrated in Transport Buffer, for 1 hour at 4°C on a platform rocker. Beads were pelleted by a brief (5 second) centrifugation at 10 000 x g, and washed 4 times with 500 µl Transport Buffer. To remove proteins from the resin the GST chimeras were cleaved with 0.3 NIH U thrombin (Sigma, Oakville, Canada) at room temperature for 15 minutes. Thrombin was inactivated after the incubation by the addition of 1 U Hirudin (Sigma, Oakville, Canada). Cleaved proteins were collected by centrifugation and the supernatant transferred to a new tube. To clear residual GST-containing proteins, 10 ul of pre-equilibrated resin was added, followed by a 30 min incubation at 4°C, and centrifugation.

For assays involving interactions between 2 or more proteins, the initial binding steps were done as above. Cleaved proteins were incubated with GST-fusions, pre-bound to the GT resin, for 1 hour, 4°C on a platform rocker. Samples were centrifuged and the

supernatant (unbound) precipitated by adding 1/10th volume of 100% trichloroacetic acid (TCA)(BDH), followed by incubation on ice for 20 minutes. Unbound fractions were then centrifuged at 4°C, 11 250 x g, for 15 minutes and washed with 0.5% TCA, followed by resuspension in SDS sample buffer. The bound fractions were washed 3 times with Transport Buffer prior to cleavage from the resin as described above and analyzed by SDS PAGE and Coomassie Blue staining.

2.12. Single stranded DNA (ssDNA) binding assay

Recombinant proteins were purified and cleaved as above. Single stranded DNA–Cellulose (ssDNA; Amersham, Cleveland, OH) was prepared by adding 1 g of ssDNA to 1 ml of 50 mM Tris/20 mM EDTA and stored at 4°C. Before use, 100 µl of ssDNA slurry was washed twice with Transport Buffer. ~7.5 µg of cleaved proteins were incubated in a 500 µl volume with ssDNA (100 µl of 50% slurry) for 1 hour, 4°C. Samples were centrifuged briefly and washed 3 times with Transport Buffer. Aliquots of the ssDNA-protein complex were made and incubated with additional protein samples prepared as described above for 1 hour, at 4°C. Samples were centrifuged briefly, the unbound fractions collected, and precipitated with TCA as above. Bound fractions were boiled with SDS sample buffer and samples analyzed by SDS PAGE and Coomassie Blue staining.

2.13. Ran binding

Protein complexes were formed as above, and separated into aliquots. Three micrograms of Ran-GTPγS, Ran-GTP, Ran-GDP, or buffer alone was added and samples were incubated for 30 minutes at room temperature. Samples were centrifuged for 5 seconds and the unbound fractions collected and processed as above. The beads were washed 3 times with Transport Buffer and bound proteins were cleaved from the resin with thrombin. Bound and unbound fractions were analyzed by SDS PAGE and Coomassie Blue staining.

2.14. GFP analysis

Wild type or mutant *S. cerevisiae* cells containing GFP-tagged proteins were grown in selective media and the GFP was visualized directly by fluorescent microscopy using a Zeiss Axioskop 2. Images were captured using a Spot camera (Diagnostic Instruments Inc., Sterling Heights, MI). In the case of temperature sensitive cells, cultures were grown overnight at 23°C, shifted to 37°C for the indicated time and examined for GFP distribution. Sample temperatures were maintained on the microscope using a heated stage (Minitube, F.R., Germany).

2.15. Shuttling assay

The shuttling assay was performed as described by (Lee et al., 1996; Stade et al., 1997) with the following modifications: *kap104-16* cells containing Nab2p-GFP were grown at 23°C overnight and then shifted to 37°C or left at 23°C in the presence or absence of 0.1 mg/ml cycloheximide for 4.5 hours. Nab2p-GFP was visualized by fluorescent microscopy.

2.16. Ran-GTP/RNA release

Kap104p-GST was expressed and purified in *E. coli* as described above. Immobilized Kap104p-GST was incubated for 1 hour with purified, thrombin-cleaved (as above) recombinant Nab4p/Hrp1p at 4°C. Complexes were then incubated with 3 µg Ran-GTP, 10 µg total yeast RNA, or 3 µg Ran-GTP + 10 µg total yeast RNA for 30 minutes at room temperature. Unbound fractions were collected TCA precipitated, while bound fractions were washed 3 times with Transport Buffer and cleaved with thrombin. Samples were analyzed by SDS-PAGE and Coomassie Blue staining.

3. Results

3.1. Kap104 binds directly to its substrates

Previous studies implementing immunopurification have shown that Kap104p isolated from yeast cytoplasm copurified with two mRNA binding proteins, Nab2p and Nab4p/Hrp1p (Aitchison et al., 1996). However, these results do not indicate whether Kap104p interacts with each substrate individually or as a trimeric complex. To directly test this, GST fusions of Kap104p, Nab2p, and Nab4p/Hrp1p were constructed, and expressed in *E.coli*. Nab2p-GST and Nab4p-GST were first immobilized on Glutathione (GT)-Sepharose and then incubated with recombinant Kap104p. It was found that Kap104p could bind directly to both Nab proteins *in vitro*, however was unable to bind GST alone (Fig 1A). The relative amounts of protein as seen by Coomassie blue staining suggest that Kap104p and Nab2p or Nab4p bind in roughly equimolar amounts, and with similar capacity, which is consistent with the findings of Aitchison et al. 1996. Also, unlike what is known for Kap95p (Enenkel et al., 1995), Kap104p binds directly to its substrates without the need of an adapter protein, such as Kap60p. Under the same conditions, Kap104p was unable to form a trimeric complex with both Nab2p and Nab4p, thus the cytoplasmic interactions previously observed were most likely two independent heterodimeric import complexes.

3.2. Identification of an RG-NLS in Nab2p and Nab4p/Hrp1p

The vertebrate orthologue of Kap104p, kap β_2, binds to its substrate at a unique sequence termed M9 present in hnRNPA1 (Pollard et al., 1996). To identify the recognition sequence required for Kap104p mediated import, we first examined the protein sequence of Nab2p. Nab2p contains two distinct RNA binding motifs: amino acid residues 200-249 are rich in arginine and glycine residues, and conforms to the consensus of an RGG box sequence characteristic of many RNP proteins, amino acid residues 249-474 contains a CCCH Zn-finger domain. In addition to these motifs, Nab2p also contains a glutamine-rich region between residues 101-172 (Kiledjian and Dreyfuss, 1992; Anderson et al., 1993; Dreyfuss et al., 1993).

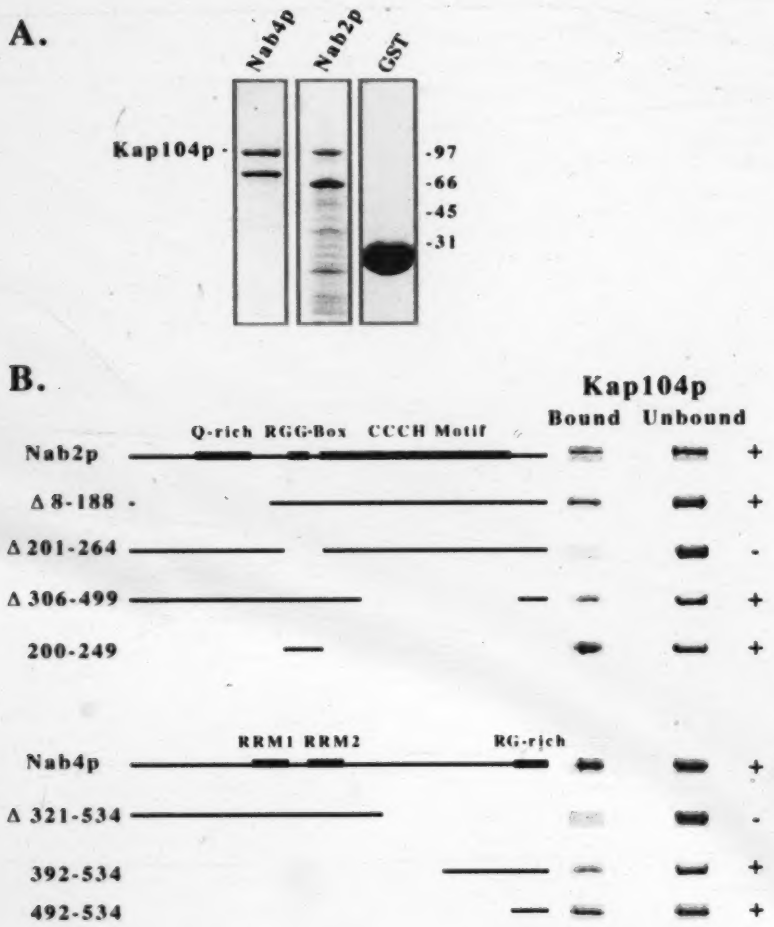
Figure 1.

Nab2p and Nab4p/Hrp1p bind Kap104p through similar RG-rich domains.

(A) GST fusions of Nab2p, Nab4p/Hrp1p (Nab4p) were expressed in *E. coli*, and immobilized on Glutathione Sepharose 4B beads to yield ~0.3 µg protein per µl of packed beads. Recombinant Kap104p (10 ng/µl) was added and incubated at 4°C for 1 hour. After washing, bound fractions were released from GT resin by cleavage with thrombin and analyzed by SDS-PAGE and Coomassie Blue staining. As a control, GST alone was bound to GT to yield ~1.5 µg protein per µl of packed beads, incubated with 10 ng/µl of recombinant Kap104p and treated as above. No detectable Kap104p bound to GST alone.

(B) Deletion constructs of Nab2p and of Nab4p/Hrp1p were expressed as GST fusions and bound to GT-Sepharose as above. The line diagram represents the regions of each protein which were expressed. Purified recombinant Kap104p was added (10 ng/µl) to each chimera and incubated for 1hour at 4°C. Bound and unbound fractions were analyzed by SDS-PAGE, followed by Coomassie Blue staining. Kap104p bound and unbound fractions are shown at the right of each line diagram.

(C) The rg-NLS of Nab2p was compared with full-length Nab4p/Hrp1p using MegAlign (Lipman-Pearson: ktuple: 2, Gap Penalty: 4, Gap Length Penalty: 12).


A.
Nab4p
Nab2p
GST
Kap104p
-97
-66
-45
-31
B.
Kap104p
Bound
Unbound
Q-rich
RGG-Box
CCCH Motif
Nab2p
Δ 8-188
Δ 201-264
Δ 306-499
200-249
RRM1
RRM2
RG-rich
Nab4p
Δ 321-534
392-534
492-534
C.
210
220
230
240
Nab2p DNSQRFTQRGGGAVGKNRRGGRGGNRGGRNNNSTRFNPLAK
Nab4p DRDRDYNHRSGG---NHRRNGRGG-RGGYNRRNNGYNPYNR
500
510
520
530

We expressed deletion constructs of these motifs as GST chimeras in *E.coli*, and tested their ability to bind Kap104p using *in vitro* binding assays (Fig.1B). Nab2p constructs lacking the Q-rich region (Δ8-188; Fig.1B) or the Zn-finger domain (Δ306-499; Fig.1B) maintained their ability to bind Kap104p. However, Nab2p lacking the arginine and glycine (RG) rich RGG box (Δ201-264; Fig.1B) failed to bind Kap104p. These findings suggest that that the RGG box of Nab2p is required for Kap104p binding. To test this hypothesis, the RGG motif was expressed as a GST fusion, and assayed for binding to Kap104p. As expected the RGG box was able to bind directly to Kap104p, demonstrating that this sequence is essential and sufficient for Nab2p binding by Kap104p. Similar results have been reported using a yeast two-hybrid screen for the Kap104p interacting domain within Nab2p. In these cases amino acid residues 198-252 (Siomi et al., 1998) and 181-251 (Truant et al., 1998) were shown to interact with Kap104p. The *in vitro* binding experiments reported here extend the two hybrid data to show a direct interaction between this region of Nab2p and Kap104p.

Using similar techniques used for Nab2p, Nab4p/Hrp1p was assayed for a Kap104p interacting region. Nab4p/Hrp1p contains two **R**NA **r**ecognition **m**otifs (RRMs) between amino acid residues 161-291 and an RG-rich carboxyl terminus (Adam et al., 1986; Query et al., 1989; Dreyfuss et al., 1993; Henry et al., 1996). As described above, the RGG-box of Nab2p is a likely candidate for being a unique type of NLS. Noting that Nab4p also contains an RG-rich region, deletion constructs of Nab4p were created and assayed for Kap104p binding. Nab4p/Hrp1p lacking the RG-rich C-terminus (Δ310-534; Fig.1B) was unable to bind Kap104p in solution binding assays. When expressed alone, the C-terminal sequence (392-534; Fig.1B) of Nab4p/Hrp1p was capable of interacting with Kap104p. Sequence comparison of Nab4p/Hrp1p with the RGG box of Nab2p revealed that the extreme carboxyl terminus (498-534) of Nab4p/Hrp1p was also RG-rich. Fig.1C shows the amino acid sequence alignment between Nab2p RGG box and the extreme C-terminus of Nab4p/Hrp1p. This C-terminus region of Nab4p/Hrp1p is similar to the RGG box of Nab2p with respects to arginine and glycine, however it does not fit the RGG box consensus sequence (Kiledjian and

Dreyfuss, 1992; Dreyfuss et al., 1993). Both the RGG box of Nab2p and the extreme C-terminus of Nab4p/Hrp1p are also similar in that they contain highly basic residues. However, while the RGG-box of Nab2p contains 6 hydrophobic residues, the C-terminus of Nab4p/Hrp1p contains no hydrophobic residues. When expressed as a GST fusion, the 37 residue C-terminus of Nab4p/Hrp1p was also able to bind Kap104p directly in solution binding assays (Fig.1B). This region may also be a candidate for a unique NLS that is important in Kap104p mediated import.

To test whether these RG-rich (arginine/glycine) sequences in Nab2p and Nab4p/Hrp1p could serve as functional NLSs, the protein sequences were tagged with two tandemly repeated GFP (**G**reen **F**lorescent **P**rotein) proteins, and visualized in living yeast cells (W303 strain) (Fig.2A). While GFP alone localized to the cytoplasm, the RG-rich domains of Nab2p and Nab4p/Hrp1p targeted GFP protein to the nucleus, as did the cNLS of SV40 large T antigen fused to the same GFP construct (Fig.2A). These findings demonstrate that these RG-rich Kap104p interacting domains can indeed function as NLSs *in vivo*. To distinguish these NLSs from cNLSs which are cargo for the Kap60p/Kap95p pathway, Nab2p and Nab4p/Hrp1p NLSs will be referred to as RG-NLSs.

Although the RG-NLSs of Nab2p and Nab4p/Hrp1p are imported into the nucleus it is unclear from the above experiment whether Kap104p mediates their localization. To address this question, the RG-NLS-GFP chimeras were transformed into *kap104-16* temperature sensitive (ts) mutant cells (Aitchison et al., 1996) and GFP localization analyzed (Fig.2B). At 23°C both RG-NLS constructs, and cNLS-GFP were localized to the nucleus as in W303 cells. However, shifting the cells to 37°C resulted in the mislocalization of both RG-NLS chimeras to the cytoplasm. In contrast cNLS-GFP distribution was not affected by the temperature shift and remained in the nucleus. At 37°C, *kap104-16* ts cells rapidly degrade Kap104p and less than 10% of cellular Kap104p is detected after 3 hours incubation time. Thus the change in cellular localization of RG-NLS-GFP was most likely due to the lack of Kap104p present for transport.

Figure 2A.

Kap104p-interacting domains of Nab2p and Nab4p/Hrp1p are imported into the nucleus. Amino acid residues 200-249 of Nab2p (Nab2p RG-NLS), 492-534 of Nab4p/Hrp1p (Nab4p RG-NLS) and the cNLS of SV40 large T antigen (cNLS), were expressed as 2XGFP chimeras in wild type (WT) W303 haploid cells. Cells were observed using differential interference contrast (DIC) (right column) and GFP was detected by fluorescent microscopy (left column). In each case the GFP signal was localized to the nucleus.

A.

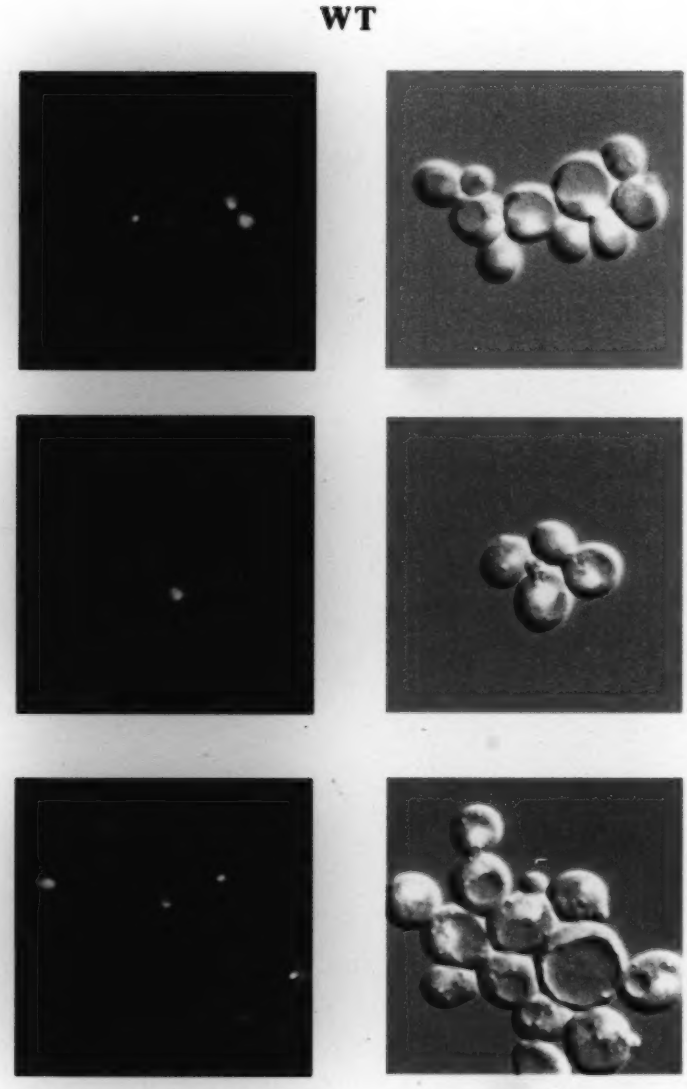
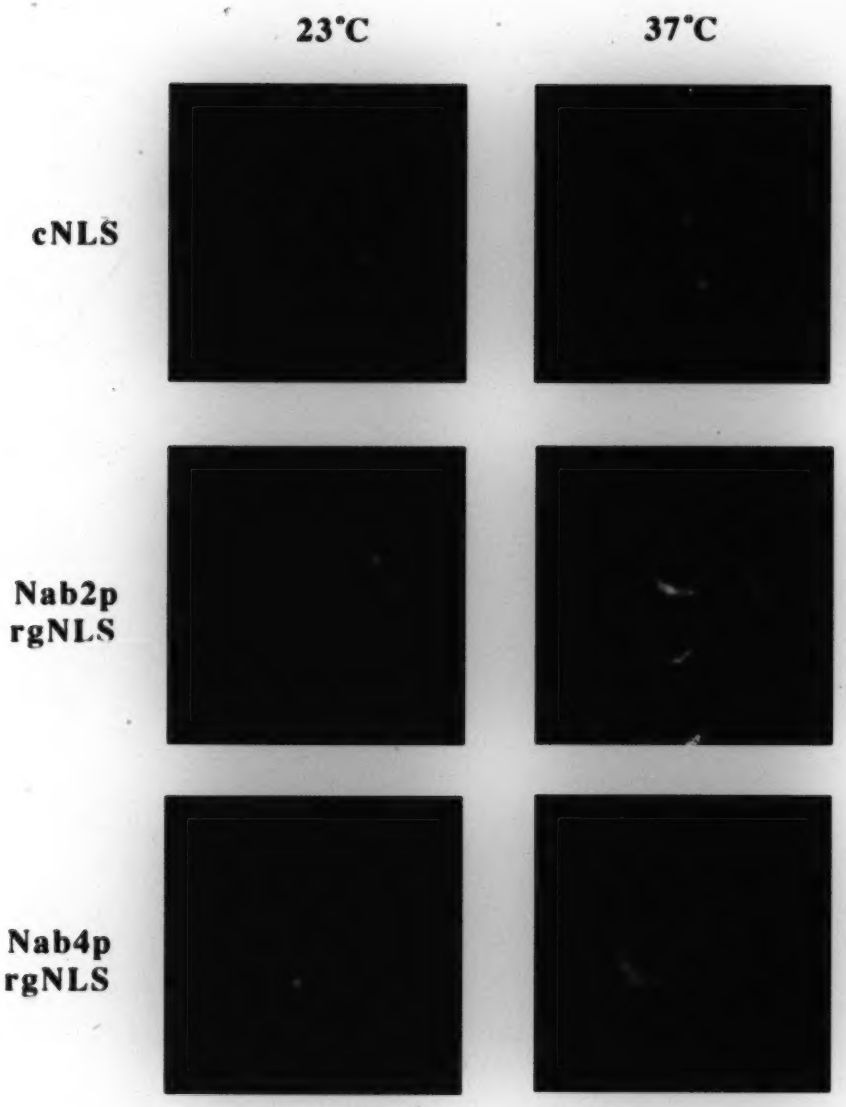


Figure 2B.

RG-NLS of Nab2p and Nab4p/Hrp1p are imported into the nucleus in a Kap104p dependent pathway. The RG-NLS-GFP and cNLS-GFP chimeras mentioned in Fig.2A were expressed in *kap104-16 ts* cells. *kap104-16* cells were maintained at 23°C or grown at 23°C and shifted to 37°C for 4.5 hours prior to examination. The GFP chimeras were visualized by fluorescent microscopy. All cells grown at 23°C maintained a nuclear GFP signal, however RG-NLS-GFP containing cells displayed a cytoplasmic signal after being shifted to 37°C. In contrast, nuclear signal was observed in cells containing cNLS-GFP at 37°C.

B.



As mentioned above the cNLS-GFP did not change in distribution after a temperature shift, suggesting that the cNLS import pathway was unaffected by the absence of Kap104p and that the two pathways are distinct and unique. Similar results were obtained when cNLS-GFP and the RG-NLS-GFP of Nab2p were transformed into cells where *KAP104* was placed under the control of a galactose (*GAL10*) inducible promoter. When grown in galactose RG-NLS-GFP was localized to the nucleus, however this distribution was shifted to the cytoplasm when cells were grown in glucose for 24 hours (Fig.3). Likewise cNLS distribution was unaffected by the glucose shift and remained nuclear (Fig.3). Shifting the cells to glucose inhibits the expression of Kap104p, thus the RG-NLS-GFP fusion protein became mislocalized to the cytoplasm due to lack of Kap104p. Taken together, the data from both *kap104-16* strains and galactose inducible *Kap104* cells demonstrate that Kap104p mediates the import of Nab2p, and Nab4p/Hrp1p through interacting with the RG-NLS domains of both proteins. However, Kap104p is not likely to be a major contributor to cNLS import pathways.

To further support the above data, cNLS-GFP and RG-NLS-GFP Nab2p were transformed into a *kap95* temperature sensitive mutant (J. Aitchison, personal communication) and assayed for GFP localization before and after a temperature shift to 37°C. At 23°C, cNLS-GFP and RG-NLS-GFP were localized to the nucleus. However after a shift to 37°C the cNLS-GFP fusion protein redistributed to the cytoplasm, while RG-NLS-GFP remained nuclear (Fig.4). These results complement the data described above by demonstrating that the cNLS and RG-NLS follow distinct pathways utilizing unique transport mechanisms.

Interestingly, like a cNLS (Stade et al., 1997), RG-NLS can be overcome by attaching a functional leucine rich nuclear export sequence (NES) to the chimera (Fig.5). In contrast, a mutant NES (nes) does not redistribute RG-NLS-GFP, and the chimera remains nuclear.

Figure 3.

Nab2p-GFP is mislocalized to the cytoplasm in the absence of Kap104p.

Full length Nab2p-GFP and cNLS-GFP were expressed in Kap104-GAL cells, and grown in galactose at 23°C. Cells were then switched to glucose (GLU) or maintained in galactose (GAL) for 24 hours. Analysis was performed as in Fig.2B. Both Nab2p-GFP and cNLS-GFP were nuclear when grown in galactose. Nab2p-GFP grown in glucose exhibited a strong cytoplasmic signal. Whereas cNLS-GFP remained nuclear in cells grown in glucose containing media.

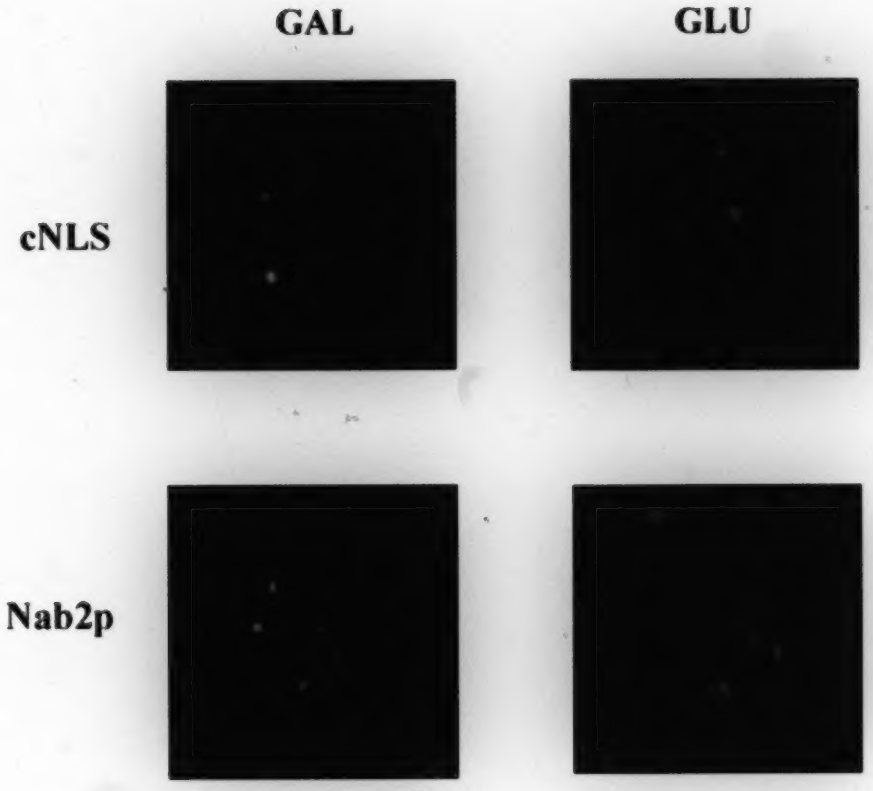

GAL
GLU
cNLS
Nab2p

Figure 4.

RG-NLS is not mislocalized in the absence of Kap95p.

RG-NLS-GFP of Nab2p and cNLS-GFP were expressed in *kap95* temperature sensitive cells. Cells were grown at 23°C, then either maintained at 23°C or shifted to 37°C for 3 hours and visualized as in Fig.2B. Cells grown at 23°C displayed nuclear signal for both RG-NLS-GFP and cNLS-GFP. RG-NLS containing cells grown at 37°C also showed a nuclear signal, whereas cNLS-GFP was localized to the cytoplasm at 37°C.

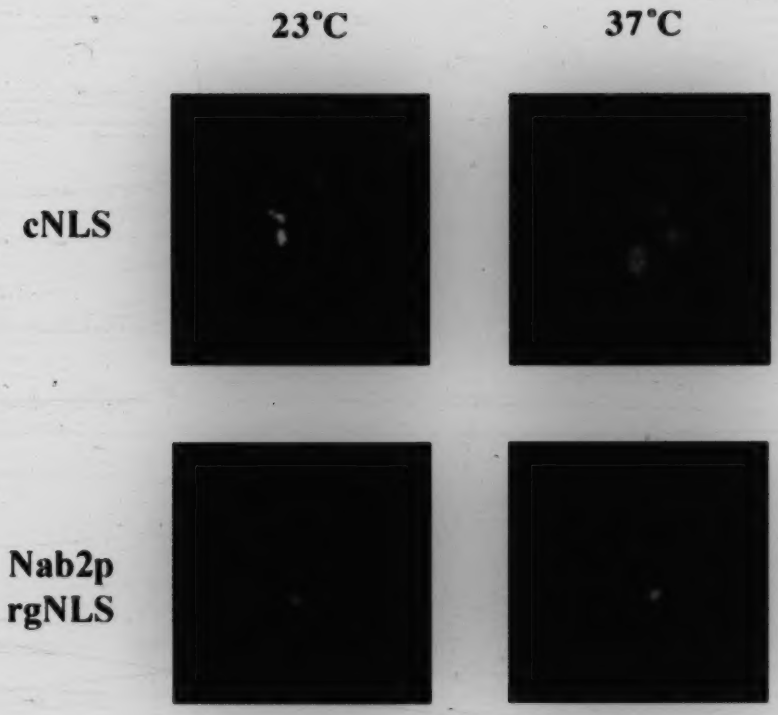
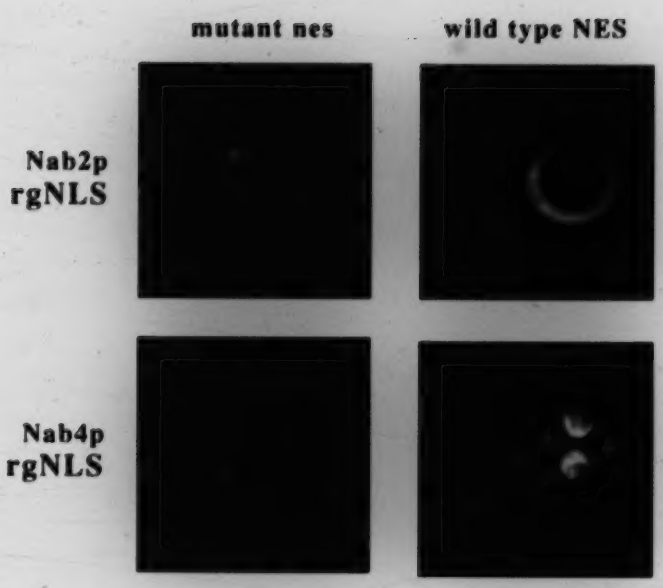

23°C
37°C
cNLS
Nab2p
rgNLS

Figure 5.

RG-NLS can be overcome by an NES *in vivo*.

RG-NLS regions of Nab2p and Nab4p were cloned upstream of a mutant (mutant nes) or a functional Nuclear Export Signal (wild type NES) followed by 2 GFP tags. GFP chimeras were visualized in wild type cells grown at 30 °C. Constructs containing a mutant nes were localized to the nucleus, and chimeras with the function NES redistributed to the cytoplasm.


mutant nes
wild type NES
Nab2p
rgNLS
Nab4p
rgNLS

These results suggest that RG-NLS is not likely to function also as an NES, contrary to the dual NLS/NES function of the M9 signal of hnRNP A1 (Michael et al., 1995; Bogerd et al., 1999).

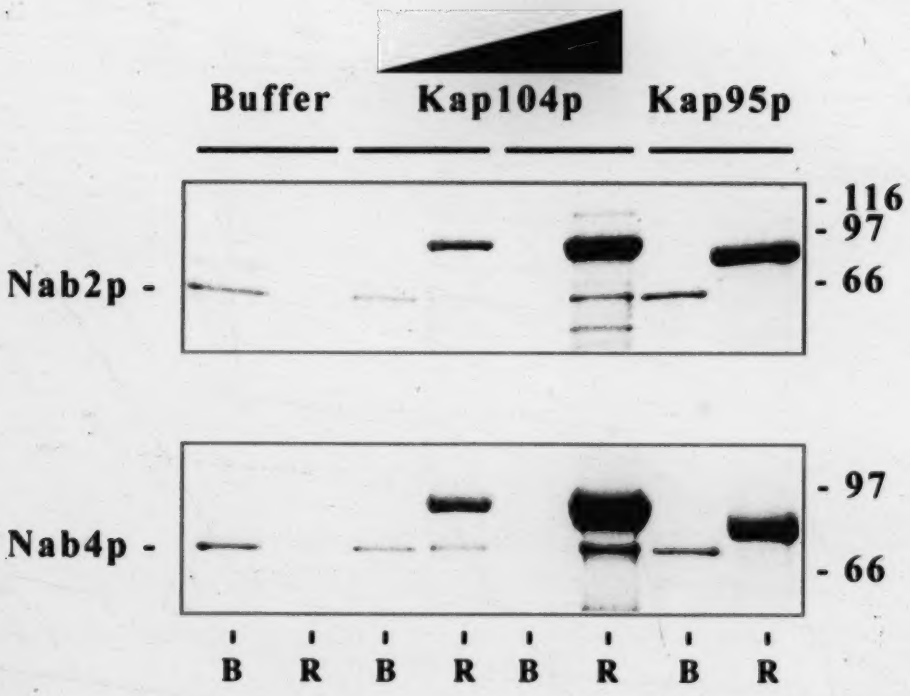
3.3. Kap104p is able to displace Nab2p and Nab4p/Hrp1p from single stranded DNA

Nab2p and Nab4p/Hrp1p have been shown to bind mRNA using UV cross-linking studies, and are believed to play a role in the export and maturation of polyadenylated mRNA (Anderson et al., 1993; Wilson et al., 1994; Henry et al., 1996; Kessler et al., 1997). As mentioned earlier, Nab2p contains two RNA binding motifs: the RGG domain and CCCH Zn-finger motif. Both of these regions are sufficient for ssDNA binding alone, however when present together the interaction is strengthened (Pinol-Roma et al., 1990; Anderson et al., 1993). Similarly, Nab4p/Hrp1p is able to bind ssDNA (Fig.6) most likely through its two RRMs (Δ321-534; Fig.1B) and the RG-rich C-terminus (Δ1-391; Fig.1B).

The overlapping RNA and Kap104p binding sites on Nab2p and Nab4p/Hrp1p possess the question of whether Kap104p is able to bind to its substrate while the RNPs are bound to mRNA. The formation of such a complex would suggest that Kap104p may contribute to mRNA export through interactions with Nab2p and/or Nab4p/Hrp1p1. To assay this *in vitro*, single stranded DNA cellulose (ssDNA) was used in place of mRNA. Previous studies have established ssDNA as a standard for isolating hnRNPs, with one of the main advantages being that it is much more stable then RNA columns (Pinol-Roma et al., 1990). Recombinant Nab2p or Nab4p/Hrp1p were immobilized on ssDNA and the complexes were treated with Kap104p or Kap95p as a control (Fig.6). Kap104p and Kap95p were not able to bind to the preformed RNP/ssDNA complex. Interestingly however, Kap104p was able to displace Nab2p and Nab4p/Hrp1p from the ssDNA column in a concentration dependent manner, as seen by a shift of Nab2p and Nab4p/Hrp1p to the unbound fractions (Fig.6).

Figure 6.

Kap104p, but not Kap95p displaces Nab2p and Nab4p/Hrp1p from ssDNA cellulose. Nab2p (upper panel) or Nab4p/Hrp1p (lower panel) bound to ssDNA (~1 μg protein / 5 μl packed resin) were incubated with Kap104p (4 and 16 ng/μl), Kap95p (14 and 10 ng/μl), or buffer alone for 1 hour at 4°C. Polypeptides in bound (B) and released (R) fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The addition of Kap104p caused the release of Nab2p and Nab4p/Hrp1p from ssDNA, as detected by the presence of Nab2p and Nab4p/Hrp1p in the unbound fractions. In contrast, Kap95p or buffer alone did not cause any detectable disruption of the Nab-ssDNA association.


Buffer
Kap104p
Kap95p
Nab2p -
- 116
- 97
- 66
Nab4p -
- 97
- 66
B R B R B R B R

Whereas, the addition of Kap95p did not have an effect on the RNP/ssDNA complex, and both Nab2p and Nab4p/Hrp1p remained in the bound fractions, suggesting that the Kap104p-induced displacement was specific (Fig.6). The relatively large amounts of Kap104p needed to produce near complete displacement from ssDNA is most likely due to the presence of other RNA binding motifs contributing to ssDNA interactions, and competition with binding sites on ssDNA cellulose. These results demonstrate that binding of Kap104p to the RG-NLS of both Nab2p and Nab4p/Hrp1p destabilizes the interaction of these RNPs with ssDNA.

In a more biological context, Kap104p binding to the RG-NLS of newly translated Nab2p and Nab4p/Hrp1p may prevent non-productive cytoplasmic Nab-RNA interactions. In addition, if Nab2p and Nab4p/Hrp1p exit the nucleus as part of an hnRNP complex bound to newly synthesized mRNA, the results above would suggest that Kap104p does not have a role in mRNA export through interactions with Nab2p and Nab4p/Hrp1p. Instead, Kap104p may be important for RNP disassembly in the cytoplasm or could be responsible for the removal of RNP proteins from mRNA to allow translation to occur.

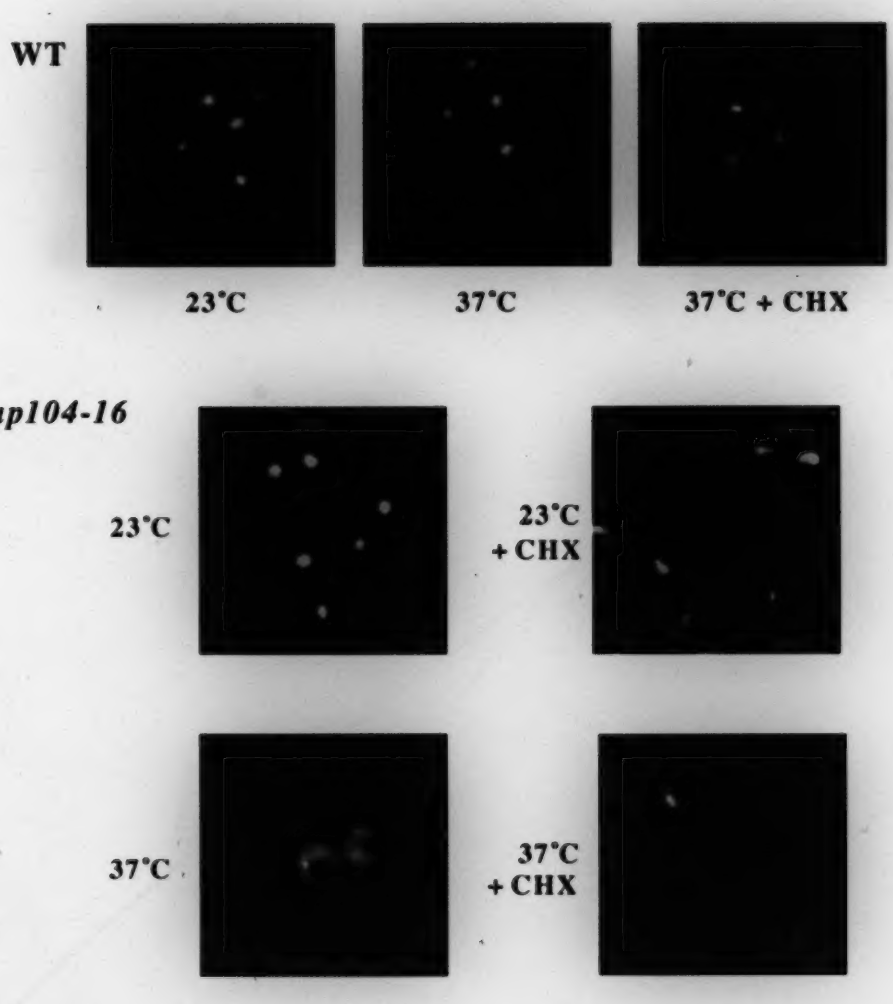
3.4. Nab2p shuttles between the cytoplasm and nucleus

If the model discussed above is valid, it would be expected that both Nab2p and Nab4p/Hrp1p exit the nucleus (with mRNA) and are subsequently re-imported for another round of mRNA export. This shuttling activity has been demonstrated for both hnRNP A1 in vertebrates (Kessler et al., 1997; Shen et al., 1998) and Nab4p/Hrp1p in yeast (Kessler et al., 1997); however, shuttling activity of Nab2p has yet to be tested. In order to determine if Nab2p is also a shuttling RNP, full length Nab2p tagged with GFP (C.Gutherie, University of California at San Francisco) was transformed into *kap104-16* ts mutants. Localization of Nab2p-GFP at 23°C was nuclear, however when shifted to the non-permissive temperature of 37°C Nab2p-GFP became cytoplasmic (Fig.7). If the cytoplasmic signal seen was due to the inability of Nab2p to be imported into the nucleus after first being exported, then protein synthesis should not be required to see a cytoplasmic signal (Lee et al., 1996; Stade et al., 1997).

Figure 7.

Nab2p shuttles between the nucleus and cytoplasm.

Nab2p-GFP chimera was expressed in wild-type (WT) and *kap104-16* cells. Cells were grown at 23°C and either maintained at 23°C or shifted to 37°C for 4.5 hours in the presence (+CHX) or absence of 0.1 mg cycloheximide per ml. Nab2p-GFP localized to the nucleus at 23°C, however upon a shift to 37°C, the GFP signal accumulated in the cytoplasm, regardless of the presence cycloheximide.


WT
23°C
37°C
37°C + CHX
kap104-16
23°C
23°C
+ CHX
37°C
37°C
+ CHX

To test this, protein synthesis was inhibited by the addition of cycloheximide to the cells prior to the temperature shift. As seen in Fig.7, the addition of cycloheximide to cultures grown at 37°C also redistributed Nab2p-GFP to the cytoplasm, indicating that Nab2p-GFP was indeed being exported from the nucleus. To ensure that the distribution shift observed was not also due in part by the addition of cycloheximide, *kap104-16* cells containing Nab2p-GFP were grown at 23°C in the presence of cycloheximide. Fig.5 shows that at 23°C cycloheximide did not cause a redistribution of the nuclear signal in *kap104-16* cells. These results suggest that cycloheximide alone did not cause Nab2-GFP to shift to the cytoplasm. However, it is still possible that a temperature shift to 37°C in addition to cycloheximide caused Nab2p-GFP redistribution. To test this, wild type cells containing Nab2p-GFP were shifted to 37°C in the presence of cycloheximide. Once again no change in nuclear GFP signal was observed (Fig.7). However it is possible that cells displaying a cytoplasmic signal were not viable, and nuclear contents were leaked to the cytoplasm after cell death. To confirm cell viability, experiments should be performed under identical conditions using cNLS-GFP as a reporter. An alternate approach would be to return shifted cells to the permissive temperature and observe whether the nuclear signal is restored. Despite this factor, the shift of Nab2p-GFP distribution was not due to the addition of cycloheximide. Since Kap104p is rapidly turned over in *kap104-16* cells grown at the non-permissive temperature, while mRNA export is unaffected (Aitchison et al., 1996), Kap104p must not have a role in mRNA export or Nab2p export from the nucleus. Instead Kap104p is most likely only involved in the import leg of the Nab2p and Nab4p/Hrp1p cycle. These results demonstrate that Nab2p, like Nab4p is able to shuttle between the nucleus and cytoplasm *in vivo*.

3.5. The role of Ran-GTP in Kap104p-mediated transport

The small GTP binding protein Ran has been shown to be a key regulator of nuclear transport (Cole and Hammell, 1998; Moore, 1998). As discussed in the Introduction, the presence of the GTP bound form of Ran in the nucleus is thought to disrupt import complexes and stabilize export complexes. Thus it acts like a molecular switch for loading and unloading substrates by karyopherins. The Kap60p/Kap95p/NLS trimeric complex has been shown to be destabilized by Ran-GTP through binding to

Kap95p (Rexach and Blobel, 1995). To investigate the role of Ran in its various nucleotide bound forms, Kap104p was first assayed for Ran binding directly. Fig.8 shows that Kap104p-GST immobilized on GT-Sepharose binds both Ran-GTP and Ran-GTPγS (non-hydrolyzable GTP analogue) but not Ran-GDP.

Previous studies have shown that Ran-GTP binds to β-karyopherins and disrupts their interaction with import substrates, these include Kap95p (Rexach and Blobel, 1995), Kap121p (Schlenstedt et al., 1997; Kaffman et al., 1998), Kap123p (Schlenstedt et al., 1997), and vertebrate kapβ_2/Trn (Izaurralde et al., 1997b; Siomi et al., 1997). To investigate if Ran-GTP has similar effects on Kap104p-Nab complexes, Nab2p and Nab4p/Hrp1p were first pre-bound to Kap104p and challenged with Ran-GTP, -GTPγS, or –GDP (Fig.9A). The addition of Ran-GTP and Ran-GTPγS stimulated a moderate release of Kap104p from both Nab2p and Nab4p/Hrp1p, as shown by a slight shift of Kap104p to the unbound fraction. However, the addition of Ran-GDP did not stimulate Kap104p release over what was observed with buffer alone under these conditions. Quantitation of these results revealed that Kap104p release was stimulated 3 fold by the presence of Ran-GTP, demonstrating that Ran must be in the GTP bound form to disrupt Kap104p from its substrates. In addition, GTP hydrolysis is not required for Kap104p release, as Ran-GTPγS was also able to release Kap104p from its substrates with similar efficiency as Ran-GTP. The effects of nucleotide alone were not assayed, thus it is not none whether the addition of a free GTP could also stimulate substrate release from Kap104p. These results are similar to studies involving Kap60p/Kap95p, however, the disassembly of the Kap60p/Kap95p/cNLS complex was nearly complete (Rexach and Blobel, 1995). To rule out dysfunctional Ran-GTP being the cause for the weaker disassembly of the Kap104p/Nab complex, Ran-GTP and Ran-GDP were incubated with pre-formed recombinant Kap60p/Kap95p complexes. Consistent with the findings of Rexach and Blobel (Rexach and Blobel, 1995), Ran-GTP, but not Ran-GDP, was able to displace the heterodimer with similar efficiency. Thus it would appear that Ran-GTP mediated displacement of the Kap104p/Nab complex is comparatively weaker than that for Kap60p/Kap95p/cNLS.

Figure 8.

Kap104p binds Ran-GTP *in vitro*.

E. coli expressed Kap104p-GST was immobilized on GT-Sepharose(~5 μg/10 μl packed resin) and incubated with purified Ran-GTPγS, Ran-GTP, Ran-GDP (3 μg/25μl) or buffer alone for 30 minutes at room temperature. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Both Ran-GTPγS and Ran-GTP bound to Kap104p, whereas Ran-GDP did not.

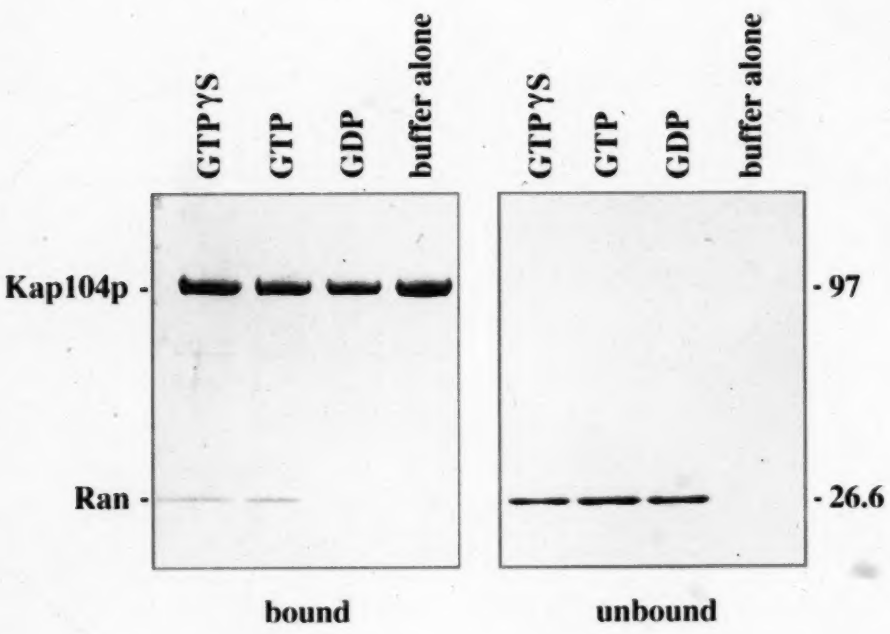

GTPγS
GTP
GDP
buffer alone
GTPγS
GTP
GDP
buffer alone
Kap104p -
Ran -
- 97
- 26.6
bound
unbound

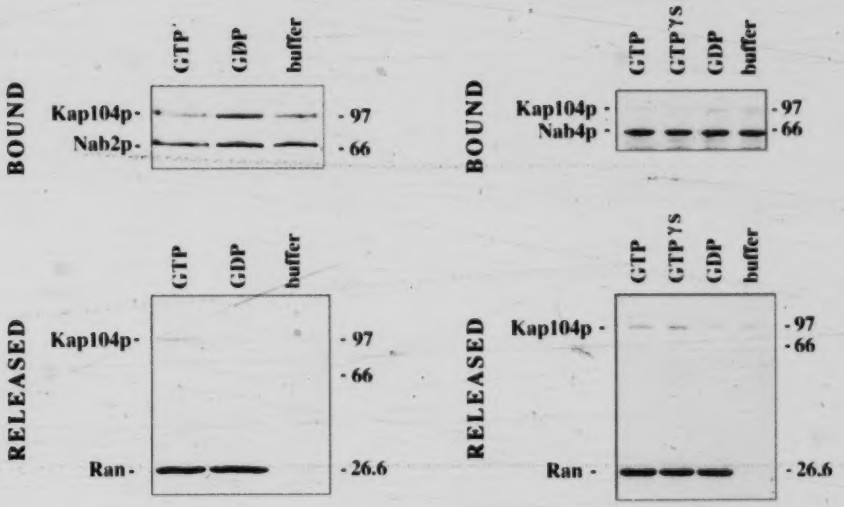
Figure 9.

Ran-GTP and RNA act cooperatively to displace Nabs from Kap104p.

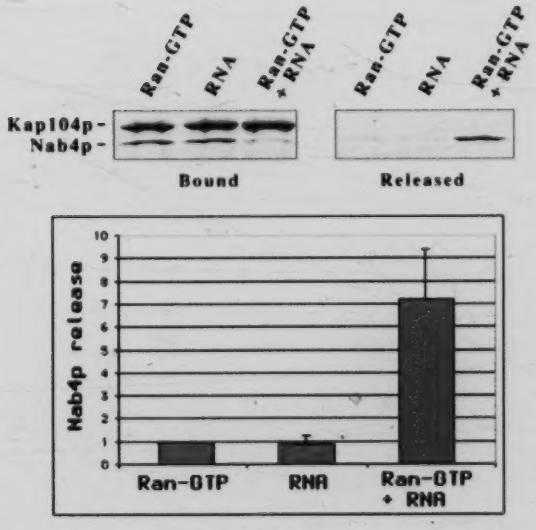
(A) Recombinant Kap104p (6 ng/μl) was incubated with immobilized Nab2p-GST (~1.5 μg/10μl packed resin) (left panel) and Nab4p/Hrp1p-GST (~2 μg/10μl packed resin) (right panel) for 1 hour at 4°C. Complexes were incubated with (3.0 μg/25μl) of Ran-GTPγS, Ran-GTP, Ran-GDP or buffer (as indicated) for 30 minutes at room temperature. Bound and released proteins were analyzed by SDS-PAGE and Coomassie Blue staining. Kap104p release was quantitated using Image-Quant software, revealing that Ran-GTPγS and Ran-GTP caused a 3-fold increase in the displacement of Kap104p from its substrate over that observed with Ran-GDP or buffer alone.

(B) Immobilized Kap104p-GST (~1 μg/10μl packed resin) was incubated with cleaved Nab4p/Hrp1p (6 ng/μl). Complexes were then incubated with 1) Ran-GTP (3.0 μg/25μl), 2) Ran-GTP (3.0 μg /25μl)+ total yeast RNA (10 μg/25μl), 3) total yeast RNA (10 μg/25 μl). Released fractions and bound fractions (after cleavage from the resin with thrombin) were analyzed by SDS-PAGE and Coomassie Blue staining. The results of quantitation of Nab4p/Hrp1p in the released fractions in three separate experiments are represented graphically. Ran-GTP mediated release was normalized to 1.0.

A.


BOUND
GTP
GDP
buffer
Kap104p
Nab2p
97
66
BOUND
GTP
GTPγS
GDP
buffer
Kap104p
Nab4p
97
66
RELEASED
GTP
GDP
buffer
Kap104p
Ran
97
66
26.6
RELEASED
GTP
GTPγS
GDP
buffer
Kap104p
Ran
97
66
26.6

B.


Ran-GTP
RNA
Ran-GTP + RNA
Kap104p
Nab4p
Bound
Released
Nab4p release
Ran-GTP
RNA
Ran-GTP + RNA

The slight release of Kap104p from Nab2p and Nab4p/Hrp1p brings up the question of what other factors are needed for more complete dissociation between the karyopherin and its substrates. It is hypothesized that Kap104p must unload its substrates in the nucleus where the RNP proteins bind mRNA. Thus a nuclear factor may participate in the release of Kap104p from Nab2p and Nab4p/Hrp1p after the import complexes enter the nucleus. Because Kap104p stimulates release of both Nab2p and Nab4p/Hrp1p from ssDNA (Fig.6), it is possible that RNA may play a role in Nab unloading from Kap104p. To test this, Kap104p-GST was immobilized on GT-Sepharose and Nab4p/Hrp1p added. The complex was then incubated with Ran-GTP, RNA, or Ran-GTP+RNA. Under these conditions, the addition of Ran-GTP or RNA alone stimulated a weak release of Nab4p/Hrp1p from Kap104p (Fig.9B). Interestingly, the combination of Ran-GTP and RNA had a cooperative effect on releasing the substrate, and nearly complete displacement was seen (Fig.9B). Quantitation of the results revealed that Ran-GTP and RNA alone stimulated approximately the same amount of release of Nab4p/Hrp1p from Kap104p. In contrast, the combination of both Ran-GTP and RNA produced release of over seven fold higher than Ran-GTP and RNA alone. This suggests that in an environment that contains RNA and high Ran-GTP concentrations, the Kap104p/Nab complex would be dissociated. Interestingly, these conditions are present in the nucleus where Ran-GTP predominates and active RNA transcription is present. Further experimentation involving the addition of RNA+nucleotide to a Kap104p import complex is needed to ensure that the displacement of Nab4p/Hrp1p was due to the combination of RNA/Ran-GTP, and not caused by RNA/GTP.

3.6. Kap104p binds to repeat containing nucleoporins

Previous studies by Aitchison et al. (1996) demonstrated that Kap104p can interact with Nup100p, Nup116p, Nup145p, and Nup57p, through blot overlay assays. However, it is unclear whether Kap104p binds these nucleoporins directly or requires additional factors present in yeast cytosol. To investigate this, Nup116p and Nup100p were expressed as fusions to maltose binding protein (MBP) in *E.coli*, and immobilized on amylose resin prior to adding Kap104p. Both nucleoporins were able to bind

Kap104p directly in roughly equimolar amounts (Fig.10A), whereas Kap104p was unable to bind to the amylose resin alone. Studies have shown that many karyopherins bind nucleoporins through repeat motifs (Iovine et al., 1995; Radu et al., 1995b; Rexach and Blobel, 1995; Aitchison et al., 1996; Stutz et al., 1996, Rout et al., 1997, Marelli et al., 1998). To determine whether this is also the case for Kap104p, the GLFG repeat region (amino acid residues 420-958) of Nup116p was expressed as a GST fusion, and assayed for Kap104p binding. Fig.10A shows that Kap104p can form a stable complex with GLFG-GST. The Kap60p /Kap95p heterodimer has also been shown to bind nucleoporins through repeat sequences. However, the heterodimer only bound to FXFG repeats of Nup1p and Nup2p, and not to the GLFG region of Nup145 (Rexach and Blobel, 1995). These results support that the interactions at the nuclear pore are different and distinct in the Kap104p and Kap95p/Kap60p pathways.

It has been shown in this thesis that Ran-GTP is able to stimulate release of Kap104p from Nab2p and Nab4p/Hrp1p, while Ran-GDP does not destabilize the transport complex. It has also been documented that Ran-GTP is able to disassemble the Kap60p/Kap95p/FXFG-Nup1p complex (Rexach and Blobel, 1995). The data for Kap60p/Kap95p suggests that Ran is needed for karyopherin displacement from nucleoporins, which may be important for karyopherin recycling. To determine whether the Kap104p/Nup116p complex is sensitive to Ran-GTP, the pre-formed complex was incubated in the presence of Ran-GTP or-GDP. Fig.10B demonstrates that Ran-GTP is able to dissociate the Kap104p/Nup116p complex, while Ran-GDP had no detectable effect on the destabilization of the complex. The significance of this observation is not yet understood, however, it seems that Ran-GTP may mediate interactions between Kap104p and nucleoporins.

Studies have shown that the cNLS/kap α/kap β import complex can bind to Nup153p *in vitro* (Shah et al., 1998). Contrary to this, incubation of cNLS/Kap60p/Kap95p with the FXFG of Nup1p resulted in release of cNLS from the import complex, leaving the karyopherin heterodimer bound to FXFG (Rexach and Blobel, 1995).

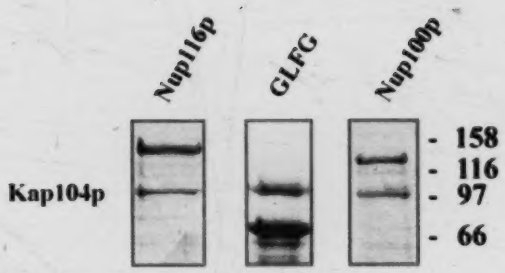
Figure 10.

Kap104p binds to nucleoporins, and is dissociated from Nup116p in the presence of Ran-GTP.

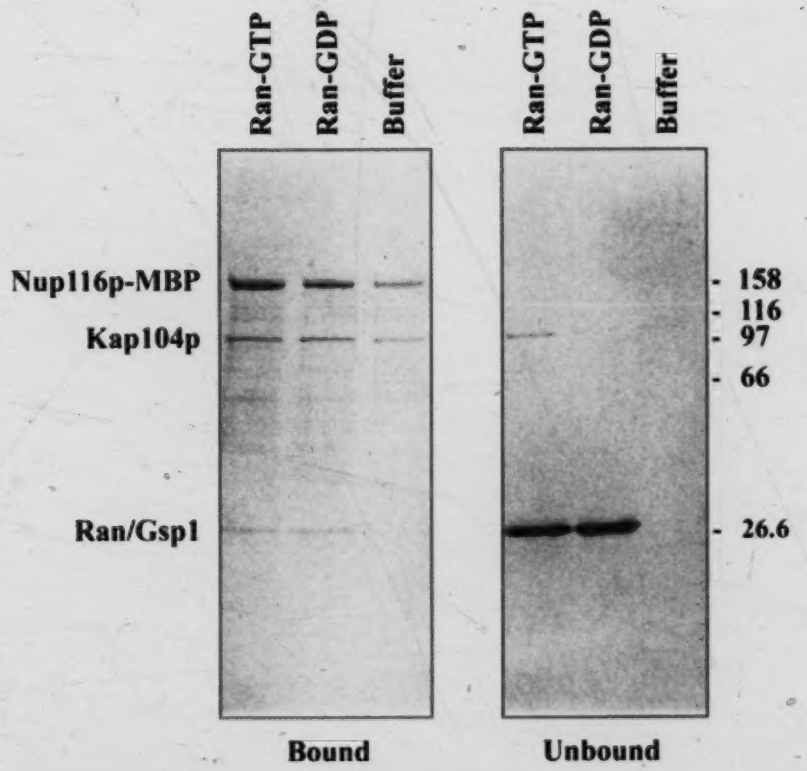
(A) Nup116p and Nup100p were expressed as fusions to maltose binding protein (MBP) and the GLFG repeat region of Nup116p was expressed as a GST fusion in *E.coli*. Recombinant Kap104p (10ng/µl) was added to immobilized Nup116p, Nup100p (1µg/10µl of packed beads) and GLFGp (5µg/10µl of packed beads), and incubated at 4°C for 1 hour. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Bound fractions shown in Panel A, demonstrate Kap104p binding to Nup116p, Nup100p, and GLFGp.

(B) Kap104p was incubated with immobilized Nup116p as described in Fig.10A. Complexes were then incubated with (3.0 µg/ 25µl) of Ran-GTP, Ran-GDP, and buffer alone for 30 minutes at 23°C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Ran-GTP was able to release Kap104p from Nup116p as seen by the appearance of Kap104p in the Unbound fraction (right). Ran-GDP and buffer alone did not cause any detectable amount of Kap104p displacement from Nup116p. The appearance of Ran-GTP and Ran-GDP in the Bound fraction was due to non-specific binding of Ran to the amylose resin.

A.


Nup116p
GLFG
Nup100p
Kap104p
158
116
97
66

B.


Ran-GTP
Ran-GDP
Buffer
Ran-GTP
Ran-GDP
Buffer
Nup116p-MBP
Kap104p
Ran/Gsp1
158
116
97
66
26.6
Bound
Unbound

Strikingly, incubation of a preformed Kap104p/Nab2p complex with immobilized Nup116p did not result in a trimeric complex. Instead, under these conditions, Nab2p did was not found in the bound fraction, however it is not entirely certain whether Kap104p is bound to Nup116p in this experiment due to the presence of degradation products of Nup116p (Fig.11A). It may seem that Nab2p is also bound to Nup116p in Fig.11A, however the band seen migrating near the mass of Nab2p is a breakdown product of Nup116p, as it is slightly higher when compared to Nab2p in the unbound lane. To confirm that the bands shown in the bound fraction are Kap104p and Nab2p, further analysis is needed using Kap104p and Nab2p specific antibodies. When recombinant Kap104p and Nab2p were incubated simultaneously with immobilized Nup116p, only Kap104p bound to Nup116p, while Nab2p remained in the unbound fraction (Fig.11B). These finding suggest that Nup116p is not involved in Kap104p mediated import or that other factors may be necessary for binding to Nup116p. Thus the exact role of nucleoporins is still unclear in Kap104p mediated transport.

3.7. Kap104p can form a trimeric complex with Ran and RanBP1p

Mutations in RanBP1 (Yrb1p) cause nuclear import and RNA export defects in yeast (Schlenstedt et al., 1995). Previous studies proposed that the binding of kap β and kap β_2 to Ran-GTP inhibits the GTPase activity of Ran (Floer and Blobel, 1996; Bischoff and Gorlich, 1997). RanBP1p is believed to inhibit the GTP stabilizing activity of kap β and kapβ_2/Trn, and stimulate the release of Ran-GTP from kap β by promoting RanGAP mediated GTP hydrolysis (Bischoff and Gorlich, 1997). As mentioned in the Introduction, a trimeric complex between kap β /RanBP1/Ran-GTP has been previously shown *in vitro* (Lounsbury and Macara, 1997). To investigate if RanBP1 also interacts with Kap104p, solution-binding assays were used. RanBP1p was expressed as a GST fusion and assayed for binding to Kap104p. Fig.12 shows that immobilized Kap104p-GST was not able to bind RanBP1p after incubation for 1 hour at 4°C. However, simultaneous addition of Ran-GTP and RanBP1p to Kap104p-GST promoted a trimeric complex between Kap104p/Ran-GTP/RanBP1p (Fig.12). This complex was likely formed by Ran-GTP interacting with both Kap104p and RBP1p simultaneously, since Kap104p and RanBP1p do not interact without Ran-GTP.

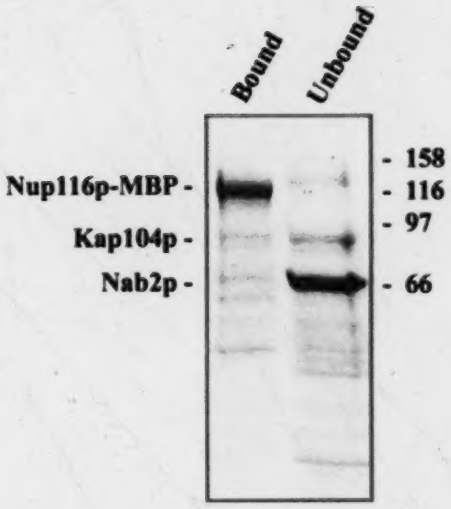
Figure 11.

Kap104p import complexes can not dock to Nup116p *in vitro*.

(A) Kap104p and Nab2p were expressed in *E.coli*, and import complexes were formed as in Fig.1A. Kap104p/Nab2p complexes were then cleaved from the GT resin and incubated with immobilized Nup116p-MBP (1 µg/10µl) for 1 hour at 4°C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The Kap104p import complex did not form a trimeric complex with Nup116p as seen by the lack of Kap104p andNab2p in the bound fraction. However it is not clear whether Kap104p is bound to Nup116p in this experiment.

(B) Recombinant Kap104p (1 µg/µl) and Nab2p (1 µg/µl) were incubated with immobilized Nup116p-MBP (1 µg/10µl packed beads) simultaneously for 1 hour at 4°C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Kap104p bound to Nup116p, however not in association with Nab2p, as Nab2p remained entirely in the unbound fraction.

A.


Bound
Unbound
Nup116p-MBP -
Kap104p -
Nab2p -
- 158
- 116
- 97
- 66

B.

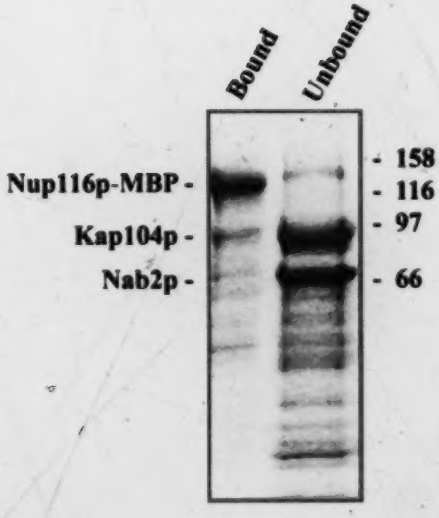
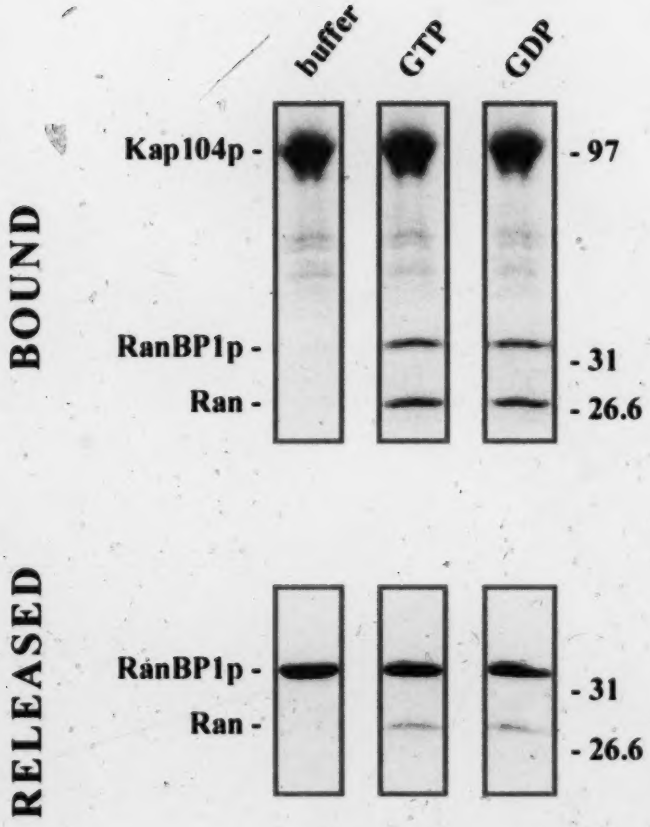

Bound
Unbound
Nup116p-MBP -
Kap104p -
Nab2p -
- 158
- 116
- 97
- 66

Figure 12.

Kap104p is able to form a trimeric complex with Ran and RanBP1p *in vitro*. Kap104p and RanBP1p were expressed as GST fusions in *E.coli*. Kap104p was immobilized on Glutathione Sepharose 4B beads to yield 0.8 µg of protein per 1 µl of packed beads, and incubated with 1) RanBP1p (5.0 µg/25 µl) and buffer, 2) RanBP1p (5.0 µg/25 µl) and Ran-GTP (3.0 µg/25 µl), 3) RanBP1p (5.0 µg/25 µl), and Ran-GDP (3.0 µg/25 µl). Bound and released fractions were analyzed by SDS-PAGE and Coomassie Blue staining. RanBP1p alone was not able to bind Kap104p in detectable amounts (left). Both the addition of Ran-GTP (middle) and Ran-GDP (right) in combination with RanBP1p resulted in the formation of a trimeric complex with Kap104p as seen in the bound fractions (upper panels).


buffer
GTP
GDP
BOUND
Kap104p -
RanBP1p -
Ran -
- 97
- 31
- 26.6
RELEASED
RanBP1p -
Ran -
- 31
- 26.6

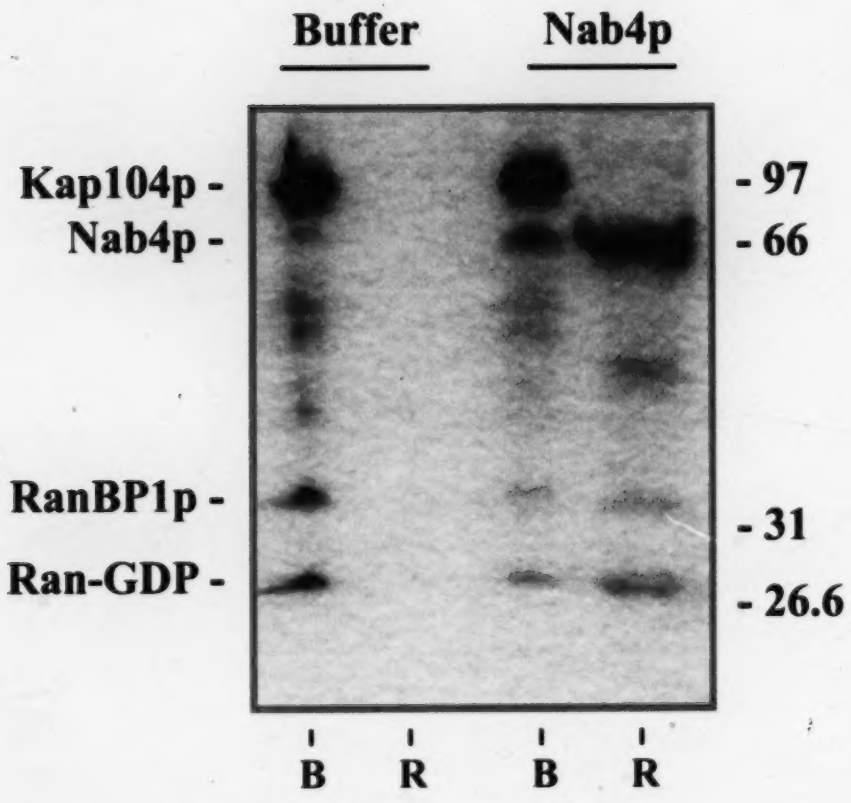
Interestingly, Ran-GDP was also able to promote the formation of a trimeric complex with Kap104p and RanBP1p. This type of association has also been observed with vertebrate kap β (Chi et al., 1997).

RanBP1p is predominately located in the cytoplasm and at the cytoplasmic rim of the nuclear envelope (Schlenstedt et al., 1995). Thus it is likely that RanBP1p interacts with Ran and Kap104p in the cytoplasm. If this is true then it may be that Kap104p binds to its substrate, RanBP1p, and Ran-GDP simultaneously in the cytoplasm. To assay this *in virto*, Kap104p/Ran-GDP/RanBP1p complexes were preformed as in Fig.12, and incubated with recombinant Nab4p/Hrp1p for 45 minutes at 23°C. Interestingly, the addition of Nab4p/Hrp1p to a preformed Kap104p/RanBP1p/Ran-GDP complex resulted in the displacement of both Ran and RanBP1p from Kap104p (Fig.13). What is the significance of these RanBP1p binding studies? It is believed that Kap104p exits the nucleus bound to Ran-GTP. Upon reaching the cytoplasmic face of the NPC, RanBP1p can form a trimeric complex with Kap104p/Ran-GTP perhaps stimulating the hydrolysis of GTP to GDP. The new trimeric complex of Kap104p/Ran-GDP/RanBP1 may remain intact until Nab2p or Nab4/Hrp1p are encountered. Upon binding of Kap104p to either Nab protein, Ran-GDP and RanBP1p would be displaced, allowing import to occur.

Figure 13.

Nab4p is able to dissociate the Kap104p/Ran/RanBP1p complex.

Kap104p/Ran-GDP/RanBP1p complexes were preformed as in Fig.12, and incubated with recombinant Nab4p/Hrp1p (0.8 μg/μl) for 45 minutes at 23°C. Bound (B) and released (R) fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The addition of Nab4p/Hrp1p resulted in the dissociation of RanBP1p and Ran-GDP from Kap104p as seen by the appearance of RanBP1p and Ran in the released fraction. Whereas buffer alone did not promote any detectable disassembly of the trimeric complex.


Buffer
Nab4p
Kap104p -
Nab4p -
RanBP1p -
Ran-GDP -
- 97
- 66
- 31
- 26.6
B
R
B
R

4. Discussion

The objective of this thesis was to characterize Kap104p-mediated import using molecular and *in vivo* approaches. Aitchison et al. (1996) established a solid foundation for further research into the *KAP104* import pathway by classifying Kap104p as a karyopherin, and by identifying potential substrates for Kap104p.

Here, Kap104p has been demonstrated to bind its substrates, Nab2p and Nab4p/Hrp1p, directly and independently *in vitro*. Deletion analysis of GST chimeras of Nab2p and Nab4p/Hrp1p revealed that these RNPs interact with Kap104p through similar arginine and glycine (RG) rich domains. These domains are both sufficient and necessary for Kap104p binding, and function as *KAP104*-dependent nuclear localization signals in yeast. To distinguish the NLS in Nab2p and Nab4p/Hrp1p from the cNLS recognized by the Kap60p/Kap95p (kap α/kap β) heterodimer, these *Kap104*-dependent NLSs are termed RG-NLSs. Kap104p directed import and heterodimer mediated import are separate, independent and unique pathways. The loss of functional Kap104p from yeast cells results in the redistribution of normally nuclear localized RG-NLS-GFP and full-length Nab2p-GFP to the cytoplasm. However, cNLS is unaffected by the absence of Kap104p (Fig.2B) and dependent on functional Kap95p (Fig.4). These findings contribute to the understanding of alternate pathways for nuclear bound proteins that do not following the cNLS system.

The Kap104p mediated import pathway is homologous to the mammailian kap β_2/Trn pathway (Pemberton et al., 1998; Siomi et al., 1998; Wozniak et al., 1998). Both pathways involve the import of RNA binding proteins, Nab2p and Nab4p/Hrp1p in yeast and hnRNP A1 in mammals. Kap104p is most similar in sequence to kap β_2 and Nab4p/Hrp1p is most similar to hnRNP A1. Interestingly, both RG-NLSs and the NLS of hnRNP A1 (M9) are rich in glycine residues. However, these NLSs are otherwise very different and contain no sequence homology. In addition, the M9 sequence is also able to function as an NES, and target normally nuclear proteins to the cytoplasm (Michael et al.,

1995; Bogerd et al., 1999), Whereas RG-NLSs do not appear to have nuclear export functions. Thus, M9 and RG-NLS are functionally different and unique from each other. This may explain why Nab2p cannot be imported by kap β_2 *in vitro* and why Kap104p is unable to import M9 (Truant et al., 1998). In line with these results, it has been reported that overexpression of kap β_2 does not complement *kap104* null cells (J.Aitchison, N.Bonifaci, and G.Blobel; unpublished results, quoted in Lee and Aitchison, 1999).

Nab2p and Nab4p/Hrp1p have been shown to be essential for cell viability (Anderson et al., 1993; Henry et al., 1996). However, deletion of *KAP104* results in a severe growth defect and temperature sensitivity, but is not lethal. Taken together these findings suggest that Nab2p and Nab4p/Hrp1p must somehow be imported into the nucleus in the absence of Kap104p. Interestingly another yeast RNP, Np13p, has also been shown to be involved in mRNA export and shuttles to between the nucleus and cytoplasm (Flach et al., 1994; Lee et al., 1996). Like Nab2p and Nab4p/Hrp1p, Np13p contains an RG-rich NLS located at the C-terminus (Senger et al., 1998) which is the recognition sequence for the karyopherin Mtr10p (Senger et al., 1998). Similar to Kap104p, the deletion of Mtr10p is also not lethal and loss of functional Mtr10p results in the mislocalization of Np13p (Pemberton et al., 1997; Senger et al., 1998). Since Kap104p and Mtr10p mediated import appear to follow similar pathways it would seem reasonable that in the absence of one, the other may substitute for the loss. However further research is needed to confirm directly that the two pathways complement each other when one of the transporters is absent. A possible method to test this would be to transform the double null cells with *KAP104* and *MTR10* separately and observe cell growth at 23°C and 37°C.

If in the absence of Kap104p, Nab2p and Nab4p/Hrp1p are imported by another karyopherin, the efficiency of this process is likely low. This assumption is based on the mislocalization of RG-NLS in *kap104-16* cells grown at the non-permissive temperature. Fig.2B shows RG-NLS-GFP localized to the cytoplasm, however there is not a prominent nuclear signal. This suggests that RG-NLS import into the nucleus is probably very minimal in the absence of Kap104p. This is supported by the severe growth defect,

demonstrated by *KAP104* null strains. An alternate mechanism for RG-NLS import in the absence of Kap104p has not yet been investigated. However, methylation of Nab2p and Nab4p/Hrp1p may be an important factor. Studies have shown that both Nab4p/Hrp1p and Np13p are arginine methylated in yeast (Siebel and Gutherie, 1996; Shen et al., 1998). This methylation is required for nuclear export, but not import. Thus it would seem that Kap104p mediated import of Nab4p/Hrp1p is independent of arginine methylation. The methylation of Nab4p/Hrp1p occurs in the nucleus by a protein called Hmt1p (Shen et al., 1998), thus newly transcribed Nab4p/Hrp1p is not likely to be methylated. This creates two separate pools of Nab4p/Hrp1p in the cytoplasm; newly translated non-methylated, and newly exported methylated. It is reasonable to hypothesize that the un-methylated pool of Nab4p/Hrp1p is considerably less abundant than the methylated population. New Nab4p/Hrp1p translation would only be required to replace the existing functional population as proteins are turned over after many rounds of transport. This is purely speculation, however it would seem energetically more economical for the cell to only replace proteins as they are degraded, rather than constantly produce new proteins for each round of transport. Thus the cytoplasmic pool of un-methylated Nab4p/Hrp1p is probably very small. It may be that this population of Nab4p/Hrp1p is the target of other karyopherins in the absence of Kap104p. However there is no data to support this hypothesis. It is still not known if Nab2p is methylated or that methylation is required for export.

The mechanism of mRNA export is still not entirely understood. It is becoming clear that newly transcribed pre-mRNA is bound by RNA binding proteins that simultaneously interact with other proteins to form hnRNP complexes. These complexes are thought to be responsible for post-transcriptional modifications and eventual export from the nucleus. Studies have shown that some hnRNPs exit the nucleus with mature mRNA, while others remain in the nucleus. Proteins that are exported from the nucleus in this manner followed by re-import are termed shuttling proteins. Nab4p/Hrp1p has been previously shown to be a shuttling hnRNP (Kessler et al., 1997), and here it has been demonstrated that Nab2p is also able to shuttle in and out of the nucleus, presumably bound to mRNA. Thus Nab2p and Nab4p/Hrp1p exit the nucleus bound to

mRNA and upon reaching the cytoplasm the hnRNP complex is dissociated for protein translation. Though not proven, Kap104p may play a role in RNP disassembly, as it destablilizes the interaction between Nab2p and Nab4p/Hrp1p with mRNA. This theory is supported by *in vitro* binding studies using ssDNA (Fig.6) which show that Kap104p is able to displace both Nab proteins from a ssDNA column by binding the RG-NLS domains. These domains, in addition to being unique NLSs, contribute to RNA binding. Thus Kap104p directly competes for binding sites with mRNA, offering a possible explanation for Nab2p and Nab4p/Hrp1p dissociation from ssDNA *in vitro*. The fact that Kap104p displaces Nab2p and Nab4p/Hrp1p from ssDNA also suggests that Kap104p is unlikely to be directly involved in mRNA export through hnRNPs, Nab2p and Nab4p/Hrp1p. Such a role would imply that although the RG-NLS overlap with the RNA binding domains, other cytoplasmic factors may contribute to Kap104p mediated displacement of Nab proteins from mRNA. This remains to be investigated.

Release of Nab2p and Nab4p/Hrp1p from mRNA by Kap104p generates an import complex that translocates through the nuclear pore. It is not known what factors in the cytoplasm contribute to Kap104p/Nab docking to the NPC. Studies have revealed that the protein p10 is essential for Kap95p/Kap60p mediated import (Moore and Blobel, 1994). This protein is thought to dock Ran-GDP to the nuclear pore and form a complex with Kap95p/Kap60p/nucleoporin/Ran where it helps regulate translocation into nucleus (Nehrbass and Blobel, 1996). Thus far there is no data for the role of p10 in Kap104p mediated import. Because of the similarities between cNLS and RG-NLS mediated transport, it seems likely that p10 may also be important for Nab2p and Nab4p/Hrp1p translocation through the pore, perhaps by bringing Ran-GDP to regions of active nuclear transport. However, further research is required to directly analyze the effects p10 may have on the Kap104p import pathway. One possible method of approaching the subject would be to observe the effects of temperature sensitive mutants of p10 on RG-NLS-GFP and Kap104p-GFP localization *in vivo*. This could be followed up with *in vitro* binding assays to determine which proteins p10 is capable of binding.

The role of nucleoporins in the Kap104p import pathway is poorly understood. Previously, it was shown that Kap104p interacts with repeat containing nucleoporins on overlay assays (Aitchison et al., 1996). Here, it is shown that Kap104p can indeed interact directly with GLFG repeat containing nucloporins Nup116p and Nup100p. The true significance of this with regards to the overall mechanism of import is still not known. However, it can be suggested that Nup116p and Nup100p do play a role in Kap104p transport. Preliminary data show that the Kap104p/Nab complex is not able to form a complex with Nup116p (Fig.11). This seems at odds with the formation of a NPC-docked transport intermediate and additional components may be required to reconstitute such a complex. It is difficult to determine from these early experiments whether these nucleoporins are indeed involved in Kap104p mediated import or just that a critical factor may be missing from the assay. Studies have shown that the cNLS/kap α/kap β complex is able to bind Nup153p in higher eukaryotes (Shah et al., 1998). However the study used *Xenopus* egg extracts as the source of transport factors and specific anti-kap α and β and anti-Nup153p antibodies for detection. Thus it can not be determined if additional factors contribute to the complex. Other studies have shown that incubation of a pre-formed complex containing cNLS/Kap60p/Kap95p with the FXFG domain of Nup1p resulted in the displacement of cNLS from the heterodimer (Rexach and Blobel, 1995). Thus a complex between nucleoporins and an NLS-containing import complex remains to be shown. An alternative hypothesis could be that Nup116p and Nup100p are not involved in Kap104p mediated import, but instead play a role in Kap104p export. However, this is not likely the case for Nup116p, as Ran-GTP is able to destabilize the Kap104p/Nup116p complex and as discussed below, the criteria for export would appear to require that a nucleoporin be able to interact with Kap104p bound to Ran-GTP.

It is well established that Ran is required for import, and that Ran-GTP is necessary for the appearance of NLS bearing proteins in the nucleus (Gorlich and Mattaj, 1996; Cole and Hammell, 1998; Moore, 1998). Like Kap95p, Kap104p binds Ran-GTP directly *in vitro*. Consistent with what is known about other import complexes, Kap104p/Nab complexes are sensitive to Ran-GTP. However unlike cNLS/heterodimer

complexes, Kap104p import complexes are only moderately destabilized in the presence of Ran-GTP. Instead, upon entering the nucleus Kap104p/Nab complexes encounter newly transcribed mRNA, which in combination with Ran-GTP promote the dissociation of the import complex. Thus Kap104p likely enters deep into the nucleus, as opposed to Kap95p which is suggested to only reach the nuclear face of the pore. This would explain the nuclear fluorescent signal seen in cells expressing Kap104p-GFP. These findings are similar to the release of Np13p from Mtr10p which also requires the cooperation of Ran-GTP and RNA (Senger et al., 1998). The requirement of Ran-GTP and RNA for substrate release imposes a mechanism for which RNA binding proteins are release at sites of active transcription. This would also prevent premature release of mRNA binding proteins before they are needed, and provide a mechanism for intranuclear protein sorting.

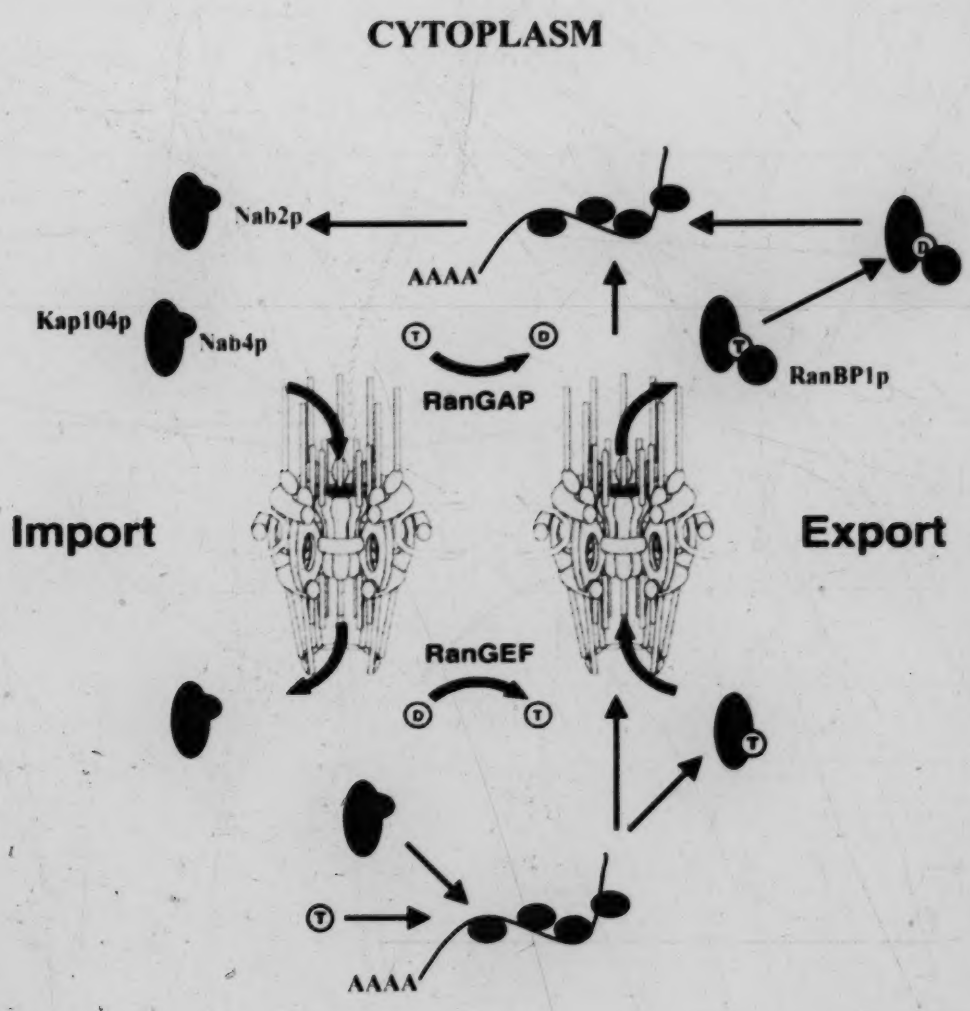
Following substrate release in the nucleus, Kap104p is likely bound to Ran-GTP. This complex then must be exported to recycle Kap104p for additional rounds of import. As mentioned above it is unclear which nucleoporins participate in Kap104p transport, and the mechanism for Kap104p exit from the nucleus is not known. Nonetheless, upon eventual export from the nucleus Kap104p emerges in the cytoplasm, presumably bound to Ran-GTP. Here, it is shown that RanBP1p can then bind the Kap104p/Ran-GTP complex and presumably promote GTP hydrolysis to GDP. The results also show this trimeric complex remains intact, though now containing Ran-GDP instead of Ran-GTP. Upon encountering Nab2p or Nab4p/Hrp1p, the Kap104p/Ran-GDP/RanBP1p complex is disassembled (Fig.13) resulting in a new import complex consisting of Kap104p/Nab. These findings suggest that the Kap104p/RanBP1p/Ran-GDP complex may be a way for the cell to prevent non-productive Kap104p shuttling by implementing a mechanism where the trimeric complex can not be imported to the nucleus.

The data collected in this thesis can be formulated into a unique model for Kap104p mediated transport (Fig.14). Although much work is still required to fully characterize this pathway, a skeletal framework has been established.

Figure 14.

Model for Kap104p-mediated nuclear import.

Kap104p binds to newly transcribed or mRNA-bound Nab2p and Nab4p/Hrp1p in the cytoplasm. These import complexes then dock and translocate through the NPC by mechanisms not yet known. Once in the nucleus, the combination of high Ran-GTP concentrations and newly transcribed mRNA cooperate to release Nab2p or Nab4p/Hrp1p from Kap104p. The Nab proteins then bind mRNA and are exported from the nucleus as part of hnRNP complexes. Kap104p exits the nucleus by an uncharacterized mechanism, however it is likely that Kap104p exits the nucleus bound to Ran-GTP. Upon reaching the cytoplasm, Kap104p/Ran-GTP forms a trimeric complex with RanBP1p, and GTP is presumably hydrolyzed. The new trimeric complex of Kap104p/Ran-GDP/RanBP1 remains intact until it encounters Nab proteins bound to mRNA. Kap104p then dissociates Nab proteins from mRNA. The binding of Kap104p to its substrate in turn releases Ran-GDP and RanBP1p from Kap104p, allowing the cycle to begin once again with the formation of an import complex.


CYTOPLASM
Nab2p
AAAA
Kap104p
Nab4p
RanGAP
RanBP1p
Import
Export
RanGEF
AAAA
NUCLEOPLASM

Kap104p is predominantly localized to the cytoplasm where it binds to its substrate Nab2p and Nab4p/Hrp1p. These RNA binding proteins exit the nucleus as part of hnRNP complexes and upon encountering Kap104p, are released from mRNA. The newly formed import complex then docks at the NPC and is translocated into the nucleus. Once in the nucleus the Kap104p/Nab complex is dissociated by newly transcribed mRNA and Ran-GTP, yielding Kap104p bound to Ran-GTP and Nab proteins bound to mRNA. Both karyopherin and substrate are then exported from the nucleus by two separate, independent pathways. In the cytoplasm, Kap104p/Ran-GTP interacts with RanBP1p and most likely RanGAP to hydrolyze GTP to GDP. A new Kap104p/Ran-GDP/RanBp1p complex is formed and remains intact until mRNA bound substrate is encountered whereupon RanBP1p and Ran-GDP are displaced from Kap104p and Nab2p or Nab4p/Hrp1p are dissociated from mRNA. The new import complex composed of Kap104p and Nab2p or Nab4p/Hrp1p then docks to the NPC reinitiating nuclear import. The details of how this complex then translocates through the NPC remains a mystery and would be an interesting focus for future work.

5. Endnotes

A version of Chapters 2, and 3 has been accepted for publication. Lee and Aitchison 1999. Journal of Biological Chemsistry. 274: 29031-29037.

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